

REMARKS

For convenience, in the present response, Applicants will refer the Examiner to disclosure in the specification by referencing the appropriate paragraph numbers of the Substitute Specification that was submitted on May 3, 2002.

Status of the claims

Upon entry of the remarks and amendments, claims 89-95, 98-104, 107-110, 113-116, 119, 121, 126-130, 133, 135, 140-144, 147, 149, 212-218, 221-227, 230-233, and 275-280 will be pending in this application. Claims 236-242, 245-256, 259-267, and 270-274 have been canceled without prejudice or disclaimer. Applicants' purpose in canceling these claims is solely to simplify, and therefore facilitate, prosecution of the instant application. Applicants assert that each of the canceled claims is fully enabled and described and therefore, satisfies the statutory requirements under 35 U.S.C. § 112. Applicants reserve the right to pursue the subject matter of the claims cancelled herein in one or more continuing applications.

The dependency of claim 110 has been amended.

Claims 212, 221 and 230 have been amended to recite "lymphocyte" in place of the word "leukocyte." Support for these amendments may be found, for example, in the specification in paragraphs [0040], [0153], [0156], [0620] and Examples 6 and 7.

Support for new (claims 279-280) directed to using Neutrokin- α polypeptides to treat common variable immunodeficiency (CVID) or Selective IgA deficiency may be found, for example, in paragraphs [0496]-[0499], [0580], [0590], [0592], and [0600].

No new matter has been added by way of amendment, and Applicants respectfully request entry of these amendments.

Request to Withdraw the "Finality" of the Office Action

The Office Action indicates that it is a "final" rejection of the pending claims. Attorneys for Applicant respectfully submit that finality is premature at this time. The Examiner's rejection under 35 U.S.C. §112, first paragraph of claims 89, 98, 126, 140, 212, 221, 230, 236, and 250 regarding the terms "leukocyte" and/or "lymphocyte" was not presented in the first Office action dated November 6, 2001. Additionally, the Examiner's

rejection under 35 U.S.C. §112, first paragraph of claims 236-263 regarding the phase "enhancement of host defenses" was not presented in the first Office action dated November 6, 2001. These rejections were neither necessitated by Applicant's amendment of the claims nor based on information submitted in an information disclosure statement filed during the period set forth in 37 C.F.R. § 1.97(c) with the fee set forth in 37 C.F.R. § 1.17(p) (See. M.P.E.P. §706.07(a)). Applicants assert that a holding of "final" rejection at this time is premature since Applicants have not yet been able to address the new ground for rejection raised by the Examiner and because a clear issue regarding this ground for rejection has not been developed between the Examiner and Applicants prior to this action (see MPEP § 706.07). Accordingly, the finality of the outstanding Office Action is improper. Applicants respectfully request that the finality of the Office Action mailed September 6, 2002, be reconsidered and withdrawn.

Rejections under 35 U.S.C. §112, first paragraph for lack of enablement

Claims 89-95, 275, 276, 98-104, 110, 277, 278, 113-117, 121, 126-130, 133, 135, 140-144, 147, 149, 212-218, 221-227, 236-242, 245-249, and 250-263 are rejected under 35 U.S.C. §112, first paragraph, for alleged lack of enablement. As the dependency of claim 110, has been amended to depend on claim 107 which is not included in this rejection, Applicants respectfully request that claim 110 be excluded from the present rejection.

More specifically, the Examiner states on page 3, lines 16-20 of Paper No. 13,

There are two points of lack of enablement under consideration addressed as follows:

- (i) how induction of B lymphocyte proliferation and enhancement of humoral response by neutrokin- α is not sufficient to provide a predictable treatment for all immunodeficiencies in general, because not all immunodeficiencies are due to same defects;
- (ii) how the scope of the administered polypeptide portions of neutrokin- α for the instant claimed treatment is not commensurate with the guidance provided in the specification.

Applicants will first address the second point of the rejection regarding the enablement of the polypeptides. Having established that the polypeptides are enabled, Applicants will then address why the claims directed to methods of stimulating lymphocyte proliferation, differentiation or survival or methods of treating immunodeficiencies, and in particular, of

treating common variable immunodeficiency (CVID) or Selective IgA deficiency, are enabled.

Enablement of polypeptides recited in the claimed methods

The Examiner expressly rejected claims 89, 98, 126, 140, 212, 221, 230, 236 and 250 under 35 U.S.C. § 112, first paragraph, for alleged lack of enablement. Each of these claims requires that the polypeptides used in the claimed methods must be able to "modulate lymphocyte, proliferation, differentiation, or survival." More specifically, the Examiner states that "[t]he specification is not enabling for the practice of these claims, because it is only the B lymphocyte proliferation that the instant neutrokin- α predictably stimulates and not proliferation of all leukocytes or lymphocytes." (Paper No. 13, page 7, lines 1-3).

Applicants respectfully disagree and traverse.

Preliminarily, Applicants point out that claims 212, 221, and 230 have been amended so as to replace the word "leukocyte" with "lymphocyte." Applicants believe that the claims were enabled prior to the amendments made herein; however, in the interest of facilitating prosecution, the above-described amendment has been made. Therefore the following discussion will be limited to the fact that (a) Neutrokin- α can modulate the proliferation differentiation, or survival of lymphocytes and (b) that one of skill in the art is capable of identifying the polypeptides that fall within the scope of the claims without undue experimentation.

(a) Neutrokin- α modulates the proliferation, differentiation, or survival of lymphocytes

The Examiner appears only to accept the enablement of methods of stimulating B cell proliferation (see Paper No. 13, page 2, line 21 to page 3, line 3) using Neutrokin- α protein due to the presence of a working example, Example 7, showing that Neutrokin- α protein stimulates B cell proliferation (see, Paper No. 8, page 4, lines 15-17). As an aside, Applicants note that Example 7 also provides a working example for modulation of B cell differentiation because administration of Neutrokin- α to mice results in an increase in the population of terminally differentiated plasma cells (CD45R(B220)^{dull}, ThB^{bright} cells) accompanied by an increase in serum immunoglobulin. Regardless, Applicants remind the Examiner that working examples are not required to

satisfy the enablement criteria of 35 U.S.C. § 112, first paragraph. (See, M.P.E.P. 2164.02, "Compliance with the enablement requirement of 35 U.S.C. § 112, first paragraph, does not turn on whether an Example is disclosed....because only an enabling disclosure is required, applicant need not describe all actual embodiments."). Applicants submit that the specification is enabling even in the absence of Example 7.

Applicants respectfully remind the Examiner that the test for enablement is whether one reasonably skilled in the art could make or use the invention, without undue experimentation, from the disclosure in the patent specification coupled with information known in the art at the time the patent application was filed. *U.S. v. Telectronics, Inc.*, 857 F.2d 778 (Fed. Cir. 1988). The enablement requirement of the first paragraph of 35 U.S.C. § 112 requires nothing more than objective enablement. How such teaching is set forth, either by the use of illustrative examples or by broad terminology, is of no importance. A specification which teaches how to make and use the invention in terms which correspond in scope to the claims must be taken as complying with the first paragraph of 35 U.S.C. § 112 unless there is reason to doubt the objective truth or accuracy of the statements relied upon therein for enabling support and it is incumbent upon the Patent Office, whenever a rejection on this basis is made, to explain why it doubts the truth or accuracy of any statement in a supporting disclosure and to back up assertions of its own with acceptable evidence or reasoning which is inconsistent with the contested statement of the presumptively accurate disclosure. *Staehelin v. Secher*, 24 USPQ2d 1513, 1516 (B.P.A.I. 1992); *In re Marzocchi*, 169 USPQ 367 (C.C.P.A. 1971); *In re Brana*, 51 F.3d 1560 at 1566 (Fed. Cir. 1995).

Applicants point out that the specification discloses that Neutrokin- α promotes lymphocyte proliferation, differentiation, and survival (see e.g., paragraphs [0153], [0154] and [0156] and Examples 6 and 7, particularly paragraphs [0850] and [0851]). These statements are presumptively accurate (See, M.P.E.P., 8th edition, section 2164.04, bottom right of page 2100-178), and have been corroborated by the use of post filing date-data. Applicants remind the Examiner that the use of post filing-date data to corroborate the enablement of a claim is in accordance with relevant case law. The Federal Circuit held in *In re Brana*, evidence dated after the filing date "can be used to substantiate any doubts as to the asserted utility since this pertains to the accuracy of a statement already in the specification." 51 F. 3d. 1560, 1567 at n19 (Fed. Cir. 1995). Such evidence "goes to prove that the disclosure was in fact enabling when filed (*i.e.*,

demonstrated utility)." *Id.*, citing *In re Marzocchi*, 439 F2d. 220 at 224 n.4. Indeed, Applicants assertions that Neutrokin-alpha stimulates lymphocyte proliferation, differentiation and survival have been substantiated by post filing-date data. In the following discussion, the literature names of Neutrokin-alpha including "BAFF" and "BLyS" will be used. Emphases are added in the following four paragraphs.

MacKay et al.¹ (cited as reference A57 on the PTO/SB-08 submitted August 20, 2001) state that "[a]t equal cell concentration, splenocytes isolated from BAFF-Tg [BAFF-transgenic] mice *survived* longer in culture when compared with control splenocytes" (p. 1703, left column); "BAFF is a powerful cytokine affecting B cells, and *has consequences for T cell* and dendritic cell status." (p. 1706, top of right column); "The presence of large germinal centers in secondary lymphoid organs of BAFF-Tg mice, *higher total T cell numbers* in the spleen and MLN [mesenteric lymph node] as well as increased proportion of both CD4 and CD8 effector T cells in the periphery, and the quality of RF isotypes strongly suggest the active participation of T cells in the immune reactions triggered in BAFF-Tg mice." (p. 1708, lower left column).

Parry et al.², (cited as reference A61 on the PTO/SB/08 submitted August 20, 2001) characterize Neutrokin-alpha as a "growth factor that *promotes B cell proliferation and differentiation*" (p. 401 right column, top).

Do et al.³, (cited as reference A50 on the PTO/SB/08 submitted August 20, 2001) teach that "[a]ttenuation of apoptosis by BLyS is not restricted to B cells after activation by antigen or CD40L, as BLyS also prolongs the *survival* of high density B cells after antigen challenge in vivo (data not shown) and naïve resting B cells in vitro (Figure 7)." (p. 962 right column, middle).

Huard et al.⁴, (provided herewith as Exhibit A) conclude that "it can be said that BAFF regulates both B and T cell activation, with an overall enhancement of *proliferation and effector responses* (Ig secretion for B cells and cytokine secretion for T cells)." (p.

¹ MacKay et al., Mice Transgenic for BAFF Develop Lymphocytic Disorders Along with Autoimmune Manifestations, *The Journal of Experimental Medicine* (1999) 190:1697-1710.

² Do et al., Attenuation of Apoptosis Underlies B Lymphocyte Stimulator Enhancement of Humoral Immune Response, *The Journal of Experimental Medicine*, (2000) 192:953-964.

³ Parry et al., Pharmacokinetics and Immunological Effects of Exogenously administered Recombinant Human B Lymphocyte Stimulator (BlyS) in Mice, *The Journal of Pharmacology and Experimental Therapeutics*, (2001) 296:396-404.

⁴ Huard et al., T cell costimulation by the TNF Ligand BAFF, *The Journal of Immunology*, (2001) 167:6225-6331.

6230, right column, middle). The induction of cytokine or immunoglobulin secretion is an indication the T or B cells have undergone a differentiation step.

Taken together these results confirm Applicants assertions that Neutrokin-alpha is able to stimulate lymphocyte proliferation, differentiation, or survival.

(b) One Skilled in the Relevant Art can Identify Polypeptides Falling Within the Scope of the Claims Without Undue Experimentation.

In Paper No. 8, the Examiner rejected all of the pending claims because it was alleged that it was unknown and/or unpredictable which fragments or variants of SEQ ID NO:2 would have the same biological activity as the full length protein. Applicants response included an explanation that the portion of the protein consisting of amino acids 134-285 of SEQ ID NO:2 has Neutrokin-alpha biological activity and that additional fragments with the same activity, namely the ability to modulate lymphocyte proliferation, differentiation or survival could readily be identified by one of skill in the art who was enlightened by the teachings of the present application. Applicants arguments were partially persuasive to the Examiner who has agreed that the region represented by amino acids 134-285 of SEQ ID NO:2 would have "the expected functionality to induce B cell proliferation." (See, Paper No. 13, page 3, lines 8-10, and see section (a) above for arguments relating to why this region is fully enabled as a region that modulates lymphocyte, proliferation, differentiation or survival.) As to the enablement of other fragments or variants of SEQ ID NO:2, Applicants arguments were not found to be persuasive. The Examiner maintained the rejection of claims 89-95, 275, 276, 98-104, 110, 277, 278, 113-117, 121, 126-130, 133, 135, 140-144, 147, 149, 212-218, 221-227, 236-242, 245-249, and 250-263 under 35. U.S.C. § 112, first paragraph, stating that:

[A]pplicants' arguments regarding scope of administered polypeptides focuses mainly on the ability of one of skill in the art to make and screen to determine which ones are operative and which ones are not. However, *it is not predictable* that polypeptides comprising... [fragments of SEQ ID NO:2 as defined in claims 89, 98, and 236, and presumably also claims 98, 221, and 250] would be able to treat immunodeficiency, or even stimulate B-lymphocyte proliferation, or differentiation or survival, because the specification fails to provide where along the entire length of SEQ ID No. 2 that changes or truncation could be made, and retain the functionality of stimulating B lymphocytes. While the skill in the art is high, *it is not predictable* that one of skill would be able to achieve peptide

variants with claimed potential, and it would require undue experimentation to screen the numerous polypeptides absent guidance and *predictable success*. (see Paper No. 13, Page 6, lines 5-17, emphases added).

Applicants respectfully disagree.

The Examiner has comprehended Applicants' previous response to this rejection that one of skill in the art can make and test polypeptide fragments and variants of SEQ ID NO:2 and determine for his or herself which ones modulate lymphocyte, proliferation, differentiation, or survival. Importantly, the Examiner has not contradicted this assertion. Rather, the Examiner intimates that the claims fail to be enabled because the specification does not indicate which fragments and variants will have the activity ("are operative") and which ones will not (are "inoperative") before the experiment is done. Applicants remind the Examiner that the law does not require that Applicants forecast the results of an experiment before it is done. Indeed, the Court of Custom and Patent Appeals has specifically cautioned that the unpredictability of the result of an experiment is not a basis to conclude that the amount of experimentation is undue in *In re Angstadt*, 537 F.2d 498 (C.C.P.A. 1976):

[If to fulfill the requirements of 112, first paragraph, an applicant's] disclosure must provide guidance which will enable one skilled in the art to determine, *with reasonable certainty before performing the reaction* whether the claimed product will be obtained, . . . then *all* "experimentation" is "undue" since the term "experimentation" implies that the success of the particular activity is *uncertain*. Such a proposition is contrary to the basic policy of the Patent Act.

Id. at 503 (emphasis in the original). As Judge Rich explained in *In re Vaeck*, 947 F2d 488, 496 (Fed. Cir. 1991), the statutory enablement requirement is satisfied if the specification "adequately guides the worker to *determine*, without undue experimentation, which species among all those encompassed by the claimed genus possess the disclosed utility" (emphasis provided). Since the disclosed or otherwise known methods of making and screening polypeptides (and fragments or variants thereof) may be used to make and then *determine*, without undue experimentation, whether a given polypeptide encompassed by the claims is able to modulate lymphocyte proliferation, differentiation or survival and therefore possesses the disclosed utility, the enablement requirement is fully satisfied. *In*

re Wands, 858 F.2d 738 (Fed. Cir. 1988); *Ex parte Mark*, 12 U.S.P.Q.2d 1904, 1906-1907 (B.P.A.I. 1989).

As to the predictability of the art, it is not so unpredictable that one skilled in the art, enlightened by the disclosure of the present application, would not be able to achieve success, *i.e.*, be able to routinely identify polypeptide fragments and variants of SEQ ID NO:2 that are capable of modulating lymphocyte proliferation, differentiation or survival. Applicants have demonstrated that a polypeptide comprising amino acids 134-285 is a functional Neutrokin- α protein. Thus each of the fragments of Neutrokin- α comprising amino acids 134-285 is fully enabled. Furthermore, fragments of Neutrokin- α that do not contain the entirety of the 134-285 region of SEQ ID NO:2 and/or variants of SEQ ID NO:2 that modulate lymphocyte proliferation, differentiation or survival are also fully enabled because assays for testing the ability of a polypeptide to modulate lymphocyte proliferation, differentiation or survival were either disclosed in the specification or were well known to those of skill in the art as of the priority date of the present application and could easily be applied to identify fragments or variants of SEQ ID NO:2 that are capable of modulating lymphocyte proliferation, differentiation or survival.

Accordingly, Applicants submit that the polypeptides recited in the claimed methods are fully enabled and respectfully request that this aspect of the rejection under 35 U.S.C. § 112, first paragraph, be withdrawn.

Enablement of the claimed methods

The other (first aspect) of the Examiner's rejection of claims 89-95, 275, 276, 98-104, 110, 277, 278, 113-117, 121, 126-130, 133, 135, 140-144, 147, 149, 212-218, 221-227, 236-242, 245-249, and 250-263 under 35 U.S.C. § 112, first paragraph pertains to the enablement of: a) methods of stimulating lymphocyte proliferation, differentiation or survival (claims 212-218, 221-227, and 230-233); b) methods of enhancing host defenses against infection (claims 236-242, 245-256, 259-267, and 270-274); or c) methods of treating immunodeficiencies, and in particular, of treating common variable immunodeficiency (CVID) or Selective IgA deficiency (claims 89-85, 275, 276, 98-104, 277, 278, 107-110, 113-121, 1126-135, and 140-149).

The enablement of claims 212-218, 221-227, and 230-233 as pertains to the ability of polypeptides of the invention to modulate lymphocyte proliferation, differentiation or

survival was discussed above (in section entitled "Enablement of Polypeptides Recited in the Claimed Methods") and will not be discussed further here so as not to overburden the prosecution history. Additionally, Applicants cancelled claims 236-242, 245-256, 259-267, and 270-274 that were directed to methods of "enhancing host defenses against infection" using Neutrokin-alpha polypeptides. Applicants' purpose in canceling or amending these claims is solely to simplify, and therefore facilitate, prosecution of the instant application. Applicants assert that each of the cancelled claims is fully enabled and satisfies the statutory requirements under 35 U.S.C. § 112. Applicants reserve the right to pursue the subject matter of the claims cancelled herein in one or more continuing applications. Thus, Applicants will only rebut the present rejection insofar as it applies to claims directed to methods of treating immunodeficiency.

Methods of treating an immunodeficiency are fully enabled under 35 U.S.C. § 112, first paragraph.

The Examiner states that, "induction of B lymphocyte proliferation and enhancement of humoral immune response by neutrokin- α is not sufficient to provide a predictable treatment for all immunodeficiencies in general, because not all immunodeficiencies are due to same defects." (Paper No, 13, page 3, lines 16-18). The Examiner illustrates her point by evaluating the enablement of claims directed to methods of treating two particular immunodeficiency diseases, namely common variable immunodeficiency (CVID, claims 275, 277, 279, 119, 133, and 147) and Selective IgA deficiency (claims 276, 278, 280, 121, 135, and 149). Applicants will first address the Examiner's arguments regarding the enablement of methods of treating CVID and Selective IgA deficiency and then address the broader issue of enablement of methods of treating immunodeficiency.

(a) Enablement of methods of treating CVID or Selective IgA Deficiency

The Examiner has argued that, "CVID is characterized by a failure of B cell differentiation, and impaired immunoglobulin production, but with variable clinical presentation." The Examiner's argument draws heavily from an English abstract of a Spanish review article on CVID by Iglesias and Matamoros which indicates that the cause of the exact defect in CVID is unknown and that several immune system abnormalities have been discovered in *in vitro* studies of samples from various patients with CVID each

of which provide distinct plausible hypotheses as to the mechanism which results in CVID patients having serum hypogammaglobulinemia. The Examiner also argued that

one type of stimulus, for example, retinoic acid alone is not sufficient to provide for B cell differentiation in order to achieve normalization of humoral immunity (abstract [of article by Saxon et al.]). Therefore, stimulating B cell proliferation alone would not provide predictable treatment for even CVID, where the defect appears to reside in an activity which neutrokin- α does not appear to affect. (Paper No. 13, page 4, lines 12-16).

In response, Applicants point out that while there may be multiple mechanistic etiologies for CVID, the failure of B lymphocytes to differentiate from antibody producing cells is "the alteration *typical* of CVI[D]" (Iglesias and Matamoros abstract, lines 7-8). Neutrokin-alpha polypeptides recited in the claimed methods have the ability to promote B lymphocyte differentiation into immunoglobulin secreting plasma cells (see section entitled "Neutrokin-alpha modulates the proliferation, differentiation, or survival of lymphocytes" above) and therefore would be expected to have an ameliorative effect in the typical case of CVID. Moreover, most of the purported additional etiologies of CVID involve a defect in lymphocyte (either B or T lymphocyte) proliferation, differentiation or survival and that therefore, the Neutrokin-alpha polypeptides utilized in the claimed methods would also be likely to have an ameliorative effect on CVID symptoms even when the etiology is not a defect in B lymphocyte differentiation to plasma secreting cells.

As regards Saxon et al.'s work on retinoic acid, Applicants submit that the proliferative effect of retinoic acid on B cells is of little probative value in the instant case, and if anything, supports a conclusion opposite to the one put forward by the Examiner. The Examiner cites Saxon et al. in support of the argument that a single B cell differentiation stimulus is not sufficient to treat CVID, whereas the abstract shows that while retinoic acid alone may not *cure* CVID patients, it did have an ameliorative effect by generating an "alteration towards normal of their immune system" and by promoting at least a partial maturation of B cells. (Saxon et al., abstract, lines 9-14 and 20-22). Thus, the work of Saxon et al. supports the conclusion that a single stimulus of B cell differentiation has use in the treatment of CVID.

The Examiner's arguments relating to the lack of enablement for claims directed to methods of treating Selective IgA deficiency are similar to the arguments outlined above

for method of treating CVID claims. The Examiner argues that the cause of Selective IgA deficiency is unknown but that

[t]he presence of normal number of IgA bearing B cells suggests that the disorder is associated with decreased synthesis or release of IgA or *impaired differentiation to IgA plasma cells*....It is true that the instant neutrokin- α could induce B cell proliferation, but this would not provide T cell responsiveness if the particular immunodeficiency is due to T cell defects. (Paper No. 13, page 4, line 20 – page 5, line 4, emphasis added.)

In response, Applicants point out to the Examiner that administration of neutrokin- α to animals results in increased serum immunoglobulin levels including increased IgA levels, and that Neutrokin- α results from in vitro studies are consistent with the interpretation that Neutrokin- α can stimulate differentiation of B cells into plasma cells. Furthermore, since Neutrokin- α has been shown to act on T cells as well as on B cells [see section entitled "Neutrokin- α modulates the proliferation, differentiation, or survival of lymphocytes" above], Neutrokin- α may still have an ameliorative effect even when the defect in Selective IgA deficiency is a T cell defect, rather than a B cell defect.

Finally, Applicants wish to bring to the Examiners attention that Human Genome Sciences is conducting Phase I clinical trials for the use of Neutrokin- α in the treatment of both CVID and Selective IgA deficiency. Section 2107.03 of the M.P.E.P., 8th edition states that

Before a drug can enter human clinical trials, the sponsor, often the applicant, must provide a convincing rationale to those especially skilled in the art (e.g., the Food and Drug Administration) that the investigation may be successful. Such a rationale would provide a basis for the sponsor's expectation that the investigation may be successful. In order to determine a protocol for phase I testing, the first phase of clinical investigation, some credible rationale of how the drug might be effective or could be effective would be necessary. Thus, as a general rule, if an applicant has initiated human clinical trials for a therapeutic product or process, Office personnel should presume that the applicant has established that the subject matter of that trial is reasonably predictive of having the asserted therapeutic utility. (emphasis in original)

Furthermore, section 2164.05(a) of the M.P.E.P., 8th edition goes on to state

Applicant should be encouraged to provide any evidence to demonstrate that the disclosure enables the claimed invention. In chemical and biotechnical applications, evidence actually

submitted to the FDA to obtain approval for clinical trials may be submitted. However, considerations made by the FDA for approving clinical trials are different from those made by the PTO in determining whether a claim is enabled. See *Scott v. Finney*, 34 F.3d 1058, 1063, 32 USPQ2d 1115, 1120 (Fed. Cir. 1994) ("Testing for full safety and effectiveness...is more properly left to the [FDA]."). Once that evidence is submitted, it must be weighed with all other evidence according to the standards set forth above so as to reach a determination as to whether the disclosure enables the claimed invention.

Exhibit C presents a summary of the non-clinical pharmacology data that Human Genome Sciences (HGS) submitted to the Food and Drug Administration in support of its proposed phase I clinical trial for the use of Neutrokin- α in the treatment of CVID. The same experiments were used to support HGS's proposed phase I clinical trial for the use of Neutrokin- α in the treatment of Selective IgA deficiency with the exception that a few additional experiments demonstrating the Neutrokin- α protein produced from an *E. coli* expression system was equivalent to Neutrokin- α protein expressed from a baculovirus expression system were also included. Exhibit C is a direct excerpt from HGS Investigational New Drug (IND) Application for the use of Neutrokin- α for the treatment of CVID. Please note that Neutrokin- α is referred to as "BLyS" in the IND.

Many of the experiments described in the IND are also disclosed in throughout the specification and particularly in Example 7. Further, Applicants submit that any additional experiments presented in the IND merely go to confirm what is disclosed in the specification and therefore serve as post filing date corroborative evidence that shows that the invention works, i.e., that the disclosure was in fact enabling when filed. (*In re Brana*, 51 F. 3d. 1560, 1567 at n19 (Fed. Cir. 1995); *In re Marzocchi*, 439 F2d. 220 at 224 n.4; M.P.E.P. § 2164.05(a)).

As those especially skilled in the art (the FDA) found experimental evidence, substantially the same as that disclosed in the present application, sufficient to enable one of skill in the art to initiate phase I clinical trials for the treatment of either CVID or Selective IgA deficiency, Applicants can see no reason for the Patent Office to disagree. Accordingly, Applicants respectfully request that this rejection, at least insofar as it applies to claims directed to methods of treating CVID or Selective IgA deficiency be reconsidered and withdrawn.

(b) Enablement of methods of treating an immunodeficiency.

The Examiner has rejected claims directed to methods of treating an immunodeficiency. Applicants would like to address the Examiner's concern that, the full scope of the claims is not enabled. Preliminarily, Applicants submit that because Neutrokin- α modulates lymphocyte proliferation, differentiation or survival, treatment with Neutrokin- α would be expected to have an ameliorative effect on a wide array of immunodeficiencies. The Examiner raised concern that certain post-filing date evidence previously cited by Applicants (e.g., Khare et al. (A55), McKay et al. (A57), and Schneider et al (A62) each cited in the revised form PTO-SB-08 submitted on August 20, 2001) suggested that Neutrokin- α treatment may induce autoimmunity in patients. Applicants note that while induction of some form of autoimmunity may be a side effect of Neutrokin- α treatment, such worry is really a safety concern that more properly falls within the oversight of regulatory agencies such as the Food and Drug Administration.

The Examiner also stated that none of the expressly cited articles indicated that Neutrokin- α "is a treatment alternative for all immunodeficiencies" (Paper No. 13, page 5, line 14). Applicants' purpose in citing these articles was to show that Neutrokin- α has biological properties, namely, modulating lymphocyte proliferation differentiation or survival, that make it useful for the treatment of immunodeficiencies. Applicants respectfully remind the Examiner that it is not a function of the claims to specifically exclude possible inoperative embodiments, and the presence of inoperative embodiments within the scope of a claim does not preclude enablement of the claim. *Atlas Powder Co. v. E. I. du Pont de Nemours & Co.*, 750 F2d 1569 at 1576 (Fed. Cir. 1984). Section 2164.08(b) of the M.P.E.P., 8th edition, instructs that "the standard is whether a skilled person could determine which embodiments that were conceived, but not yet made, would be inoperative or operative with expenditure of no more effort than is required in the art." As pointed out by the Federal Circuit, for enablement, "there must be sufficient disclosure, either through illustrative example or terminology, . . . to teach those of ordinary skill how to make and how to use the invention as broadly as it is claimed. This means that the disclosure must adequately guide the art worker to determine, without undue experimentation, which species among all those encompassed by the claimed genus possess the disclosed utility." *In re Vaack*, 947 F2d 488, 496 (Fed. Cir. 1991). Applicants submit that one skilled in the art would be able to identify without undue experimentation, the operative embodiments. Applicants submit that one of skill in the art, on the basis of

the knowledge of the biological activities of Neutrokin- α coupled with the knowledge of the defects in different immunodeficiencies is capable of determining which embodiments are likely to be operative and which ones are not.

Applicants submit that because the specification discloses that Neutrokin- α modulates proliferation, differentiation and survival of lymphocytes, and that therefore Neutrokin- α would be useful to treat the broad class of immunodeficiencies, claims 89-95, 275, 276, 98-104, 110, 277, 278, 113-117, 121, 126-130, 133, 135, 140-144, 147, 149, 212-218, 221-227, 236-242, 245-249 and 250-263 are fully enabled. Accordingly, Applicants respectfully request that this rejection under 35 U.S.C. § 112, first paragraph be reconsidered and withdrawn.

Rejections under 35 U.S.C. §112, first paragraph for lack of written description

Claims 89-95, 98-104, 107-110, 113-116, 119, 121, 126-130, 133, 135, 140-144, 147, 149, 212-218, 221-227, 236-242, 245-256, 259-267 and 270-278 stand rejected under 35 U.S.C. §112, first paragraph by the Examiner for alleged lack of written description.

More specifically, the examiner states that:

The single disclosed example of SEQ ID No.2 and the extracellular domain are not a representative of the entire claimed genus. Although the applicants assert that the types of changes in generating the variants are routine in the art and that methods of identifying active variants are provided, the specification and the claims do not provide any description as to what changes should be made and where to retain the activity, i.e., where the critical structural features reside. (Paper No. 13, page 8, lines 5-10.)

Applicants respectfully disagree.

Preliminarily, Applicants point out that the Examiner that the Examiner has already acknowledged proteins comprising amino acids 134-285 as fully enabled and described. Thus, Applicants believe that claim 230 as amended herein, drawn to "[a] method of stimulating lymphocyte proliferation, differentiation or survival comprising administering to an individual, a therapeutically effective amount of a protein **consisting of an amino acid sequence of amino acid residues 134-285 of SEQ ID NO:2**" should be excluded from this rejection.

Applicants have previously set forth the legal standard for determining compliance with the written description requirement of 35 U.S.C. §112, first paragraph (see,

Applicants Amendment and Response under 37 C.F.R. 1.111 submitted May 3, 2002). Essentially, as stated in M.P.E.P. § 2163.02, "[a]n objective standard for determining compliance with the written description requirement is, "does the description clearly allow persons of ordinary skill in the art to recognize that he or she invented what is claimed." *In re Gosteli*, 872 F.2d 1008, 1012, 10 USPQ2d 1614, 1618 (Fed. Cir. 1989)."

Applicants have also explained to the Examiner that the one of skill in the art is capable of making fragments or variants Neutrokin- α that modulate lymphocyte proliferation, differentiation or survival without undue experimentation. The Examiner does not disagree with Applicants assertion that making Neutrokin- α fragment or variant proteins or testing the activity of such fragments and variants is routine for one of skill in the art. Rather, the Examiner finds fault with Applicants disclosure because Applicants have not expressly disclosed the complete list of functional and non-functional Neutrokin- α fragments or variants.

Applicants reiterate that the law does not require such a full disclosure and refer the Examiner to section entitled "One Skilled in the Relevant Art can Identify Polypeptides Falling Within the Scope of the Claims Without Undue Experimentation" above. Furthermore, the Applicants refer the Examiner to Example 14 of the *Synopsis of Application Written Description Guidelines* Available from the USPTO website at <http://www.uspto.gov/web/menu/written.pdf>. The scenario in Example 14 closely parallels that of the current application.

In Example 14 of the *Synopsis of Application Written Description Guidelines*, the specification discloses the sequence of a protein that has a given functional activity, namely, catalyzing an enzymatic reaction. "The specification also contemplates but does not exemplify variants of the protein...The specification indicates that procedures for making proteins with substitutions, deletions, insertions and additions is routine in the art and provides an assay for detecting the...activity of the protein." *Id.* at page 53. The specification claims a protein comprising variants of the disclosed protein that are at least 95% identical to SEQ ID NO:3 and are capable of catalyzing the enzymatic reaction.

The USPTO analysis of the scenario in Example 14 is reproduced below:

There is actual reduction to practice of the single disclosed species. The specification indicates that the genus of proteins that must be variants of SEQ ID NO:3 does not have substantial variation since all of the variants must possess the specified catalytic activity and must have at least 95% identity to the reference sequence, SEQ ID NO:3. The single species disclosed

is representative of the genus because all members have at least 95% structural identity with the reference compound and because of the presence of an assay which applicant provided for identifying all of the at least 95% identical variants of SEQ ID NO:3 which are capable of the specified catalytic activity. One of skill in the art would conclude that applicant was in possession of the necessary common attributes possessed by the members of the genus.

Conclusion: The disclosure meets the requirements of 35 USC §112 first paragraph as providing adequate written description for the claimed invention.

If one merely substitutes "SEQ ID NO:3" with SEQ ID NO:2 of the present application and replaces "the specified catalytic activity" with the "specified ability to modulate lymphocyte proliferation, differentiation or survival," one is inevitably lead, by virtue of the reasoning set forth by the USPTO, to the conclusion that claims 89, 98, 126, 140, 212, 221, 230, 236 and 250 meet the written description requirements of 35 U.S.C. § 112, first paragraph. Accordingly, Applicants respectfully request that this rejection be reconsidered and withdrawn.

Lastly, the Examiner contends that the written description support for certain amendments related to "treating an immunodeficiency", treating "CVID", treating "Selective IgA deficiency" or modulating lymphocyte proliferation, "differentiation or survival" made to the claims in the Amendment and Response submitted May 3, 2002 was inadequate "because the description set forth in the specification as support for the new limitations is by way of contemplation and is prophetic, rather than showing that the Applicants are in possession of the claimed invention at the time of filing." (Paper No. 13 page 8, lines 17-18.

Applicants respectfully disagree and remind the Examiner that working examples are not required and that the teachings on the specification may also be set forth in broad terminology rather than illustrative examples. Furthermore, statements in the specification are to be taken as presumptively true unless there is reason to doubt their accuracy. *Stahelin v. Secher*, 24 USPQ2d 1513, 1516 (B.P.A.I. 1992); *In re Marzocchi*, 169 USPQ 367 (C.C.P.A. 1971); *In re Brana*, 51 F.3d 1560 at 1566 (Fed. Cir. 1995).

Moreover, Applicants have defended the objective truth of their assertions (see entire section entitled "Rejections under 35 U.S.C. § 112, first paragraph for lack of enablement." above). Thus, the issue of whether the support in the specification for the amendments submitted May 3, 2002 was "contemplative" or "prophetic" is immaterial.

Accordingly, Applicants respectfully request that this rejection under 35 U.S.C. § 112, first paragraph be reconsidered and withdrawn.

CONCLUSION

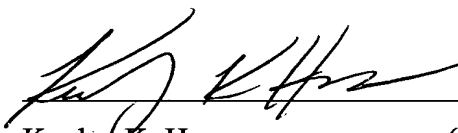
Applicants respectfully request that the amendments and remarks of the present Amendment be entered and made of record in the present application.

In view of the foregoing remarks, applicants believe that this application is now in condition for allowance. An early Notice of Allowance is earnestly solicited. If, in the opinion of the Examiner, a telephone conference would expedite prosecution, the undersigned can be reached at the telephone number indicated below.

Finally, if there are any fees due in connection with the filing of this paper, please charge the fees to Deposit Account No. 08-3425.

Respectfully submitted,

Date: March 5, 2003



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Application of: **Yu, et al.**

Application Number: 09/589,285

Group Art Unit: 1646

Filed: June 8, 2000

Examiner: Bunner, Bridget

Title: **Methods of Treatment of Immune
System Related Disorders Using
Neutrokin-alpha (as amended)**

Atty. Docket No. PF343P3C4

VERSION WITH MARKINGS TO SHOW CHANGES MADE

Amendments to the application are shown in bold-faced text with insertions indicated with underlining and deletions indicated by strikeout:

In the Claims:

Claims 236-242, 245-256, 259-267 and 270-274 have been cancelled.

Claims 110, 212, 221 and 230 were amended as shown below.

Claims 279 and 280 were added.

110. (Once Amended) The method of claim ~~98~~ 107 wherein said protein is labeled.

212. (Twice Amended) A method of stimulating lymphocyte leukocyte proliferation, differentiation or survival comprising administering to an individual, a therapeutically effective amount of a protein comprising an amino acid sequence selected from the group consisting of:

(a) the amino acid sequence of amino acid residues n to 285 of SEQ ID NO:2, where n is an integer in the range of 2-190;

(b) the amino acid sequence of amino acid residues 1 to m of SEQ ID NO:2, where m is an integer in the range of 274-284; and

(c) the amino acid sequence of amino acid residues n to m of SEQ ID NO:2, where n is an integer in the range of 2-190 and m is an integer in the range of 274-284;

wherein the polypeptide having said amino acid sequence modulates lymphocyte proliferation, differentiation, or survival.

221. (Twice Amended) A method of stimulating **lymphocyte leukocyte** proliferation, differentiation or survival comprising administering to an individual, a therapeutically effective amount of a protein comprising a first amino acid sequence that is 95% or more identical to a second amino acid sequence selected from the group consisting of:

(a) the amino acid sequence of amino acid residues n to 285 of SEQ ID NO:2, where n is an integer in the range of 2-190;

(b) the amino acid sequence of amino acid residues 1 to m of SEQ ID NO:2, where m is an integer in the range of 274-284; and

(c) the amino acid sequence of amino acid residues n to m of SEQ ID NO:2, where n is an integer in the range of 2-190 and m is an integer in the range of 274-284;

wherein the polypeptide having said first amino acid sequence modulates lymphocyte proliferation, differentiation, or survival.

230. (Twice Amended) A method of stimulating **lymphocyte leukocyte** proliferation, differentiation or survival comprising administering to an individual, a therapeutically effective amount of a protein consisting of an amino acid sequence of amino acid residues 134-285 of SEQ ID NO:2.

T Cell Costimulation by the TNF Ligand BAFF¹

Bertrand Huard,^{2*} Pascal Schneider,[†] Davide Mauri,[‡] Jürg Tschopp,[†] and Lars E. French*

The TNF ligand family member B cell-activating factor belonging to TNF family (BAFF, also called Blys, TALL-1, zTNF-4, or THANK) is an important survival factor for B cells. In this study, we show that BAFF is able to regulate T cell activation. rBAFF induced responses (thymidine incorporation and cytokine secretion) of T cells, suboptimally stimulated through their TCR. BAFF activity was observed on naive, as well as on effector/memory T cells (both CD4⁺ and CD8⁺ subsets), indicating that BAFF has a wide function on T cell responses. Analysis of the signal transduced by BAFF into T cells shows that BAFF has no obvious effect on T cell survival upon activation, but is able to deliver a complete costimulation signal into T cells. Indeed, BAFF is sufficient to induce IL-2 secretion and T cell division, when added to an anti-TCR stimulation. This highlights some differences in the BAFF signaling pathway in T and B cells. In conclusion, our results indicate that BAFF may play a role in the development of T cell responses, in addition to its role in B cell homeostasis. *The Journal of Immunology*, 2001, 167: 6225–6231.

Full activation of T lymphocytes requires at least two signals transmitted by an APC. The first one is mediated upon recognition of the antigenic peptide-MHC complex by the TCR and is called signal 1. The second one is mediated upon recognition of molecules expressed constitutively by professional APCs and is called signal 2 or costimulatory signal (1). The number of molecules providing T cell costimulation has considerably increased over the last few years, so that families, according to protein structure, can be defined. To date, the more important T cell costimulatory molecules are found in the Ig and TNF superfamilies.

Members of the TNF superfamily have pleiotropic biological functions. These molecules are involved in organogenesis, tissue homeostasis, inflammation, and immunity (2). For immune responses, TNF molecules have been implicated in both phases, the induction and the down-regulation, of a response. Involvement in the induction phase is explained by expression of TNF receptors with stimulatory activities on key immunological players, such as monocytes/dendritic cells B and T lymphocytes. At the T cell level, TNF receptors with costimulatory functions are expressed, and their respective ligands are found on professional APCs. These T cell stimulatory TNF receptor/ligand pairs are OX40/OX40L (3), 4-1BB/4-1BBL (4), CD27/CD70 (5), and the recently described herpesvirus entry mediator/LIGHT (6, 7).

B cell-activating factor belonging to TNF family (BAFF³; Blys, TALL-1, zTNF-4, THANK) is a recently identified member of the

TNF ligand family (8–11), expressed in monocytes/dendritic cells and T cells. BAFF has been described as a potent survival factor for B cells (8, 9, 12). In this study, we provide evidences that BAFF stimulates T cells, and characterize the activatory signal delivered by BAFF to T cells.

Materials and Methods

Cells and reagents

Buffy coats from healthy donors were prepared at the Geneva transfusion center. PBMCs were obtained after Ficoll-Paque gradient centrifugation, and T cells were purified by immunomagnetic depletion with an anti-CD19 (J4.119; Immunotech, Marseille, France), an anti-CD14 (RMO 52; Immunotech), an anti-MHC class II (IVA12; American Type Culture Collection (ATCC), Manassas, VA), and an anti-CD56 (B159; BD PharMingen, San Diego, CA). The cells were routinely 95% CD3⁺. Purified CD4⁺ and CD8⁺ T cells were obtained by adjunction to this mixture of an anti-CD8 (OKT8; ATCC) and of an anti-CD4 (OKT4; ATCC), respectively. Purity >98% was observed by flow cytometry analysis after staining with a PE-conjugated anti-CD4 (RPA-T4) and anti-CD8 (HIT8a), both from BD PharMingen. Memory and naive T cells were further purified from resting T cells by depletion with an anti-CD45RA (ALB11; Immunotech) and an anti-CD45RO (UCHL1; BD PharMingen). Depletion was controlled by flow cytometry analysis after staining with a PE-conjugated anti-CD45RA (HI100; BD PharMingen) and anti-CD45RO (UCHL1; BD PharMingen). The naive fraction contained routinely 95% or more CD45RA⁺ cells with <2% CD45RO⁺. The memory fraction contained routinely 95% CD45RO⁺ cells. Dully stained CD45RA⁺ cells, from 25 to 40%, depending on the donors, were also found present in this latter fraction. PHA-stimulated T cells were obtained by stimulating total PBLs for 10 days with 1 µg/ml PHA (Sigma-Aldrich, St. Louis, MO) and 100 IU/ml IL-2 (a former gift from Roussel Uclaf, Romainville, France). They were used after a resting period of 10 days. The MART-1-specific CD8⁺ T cell clone, LT12, was kindly provided by Dr. F. Faure (Paris, France).

The anti-CD25, B1.49.9 (Immunotech), was used to stain for CD25 expression. Inhibition of IL-2-dependent proliferations was performed with the anti-CD25, Mar 93, kindly provided by Dr. P. Romero (Epalinges, Switzerland).

Soluble forms of rTNF ligands and receptors were obtained from Apotech Biochemicals (Epalinges, Switzerland). Soluble forms of the following human ligands were used: BAFF (aa 83–285), Fas ligand (FasL; aa 103–281), and LIGHT (aa 89–240). These ligands were flag tagged at their amino-terminal part and purified on agarose-M2 gel (Sigma-Aldrich). rBAFF was produced in bacteria. rLIGHT and rFasL were produced in HEK 293. Soluble forms of the following human receptors were used: B cell-maturing Ag (BCMA; aa 2–54), TACI (aa 2–118), Fas (aa 7–154), and TNF-related apoptosis-inducing ligand-R1 (aa 24–239). These receptors were fused at their carboxyl-terminal part with the C region of a human IgG1. These molecules were produced in HEK 293 and purified on protein A-Sepharose (Amersham Pharmacia, Piscataway, NJ). Endotoxin levels of the purified molecules were <0.1 ng/µg purified proteins, as assessed with

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³ Abbreviations used in this paper: BAFF, B cell-activating factor belonging to TNF family; APRIL, a proliferation-inducing ligand; BCMA, B cell maturation Ag; FasL, Fas ligand; PI, propidium iodide; SI, stimulation index; TACI, transmembrane activator and calcium modulator and cyclophilin ligand interactor.

the QCL-1000 kit, according to manufacturer's instructions (BioWhittaker, Walkersville, MD).

T cell stimulation assays

T cells were activated with anti-CD3 (OKT3; ATCC) or anti-TCR (BMA 031; Immunotech) immobilized on plastic surfaces. Culture medium was RPMI 1640 supplemented with sodium pyruvate, glutamine, HEPES, and 10% heat-inactivated FCS (Life Technologies, Basel, Switzerland). Soluble anti-CD28, CD28.2 (a kind gift from Dr. D. Olive, Marseille, France), and 9.3 (a kind gift from Dr. C. June, Philadelphia, PA) were used in some experiments. Optimal concentrations of these anti-CD28 were assessed before use. Immobilization of Abs was performed overnight at 4°C in PBS. Unbound Abs were washed once, followed by immobilization of the indicated TNF ligands for 4 h at 37°C. Unbound materials were washed three times, and T cells were added at 1×10^5 cells/well (U-bottom, 0.2 ml final vol). Proliferation was assessed after 72 h by [3 H]thymidine (Hartmann Analytic, Braunschweig, Germany) incorporation for the last 18 h.

Cytokine secretion was assessed in the supernatant of activated cells with a sandwich ELISA for IL-4, IFN- γ , IL-5, and IL-13 (R&D Systems, Minneapolis, MN). TNF- α and IL-2 secretions were assessed with the sensitive cells WEHI.13 and CTLL2, respectively. Briefly, WEHI.13 cells (3×10^4) were incubated in 50 μ l of medium containing 2 μ g/ml actinomycin D (Sigma-Aldrich). After 2 h, 50 μ l of supernatant was added. Twenty-four hours later, cell viability was assessed with a WST-1-based colorimetric assay according to manufacturer's instructions (Boehringer Mannheim, Mannheim, Germany). TNF- α concentrations in cell supernatants were calculated from a standard curve obtained with purified rTNF- α (Apotech Biochemicals). A total of 4×10^3 CTLL2 cells was incubated with 1 vol cell supernatants for 24 h (final vol, 0.1 ml). [3 H]Thymidine was then added for 18 h to assess the IL-2-dependent CTLL2 proliferation. IL-2 concentrations in cell supernatants were calculated from a standard curve obtained with purified rIL-2. For IL-4, IL-5, IL-13, IFN- γ , and TNF- α detections, T cells were stimulated at 0.5×10^6 /ml. For IL-2 detection, T cells were stimulated at 2.5×10^6 /ml.

Cellular staining and flow cytometry

T cells were labeled with 250 μ M CFSE according to manufacturer's instructions (Molecular Probes, Leiden, The Netherlands). Annexin stainings were performed, according to the manufacturer's instructions (BD Phar-

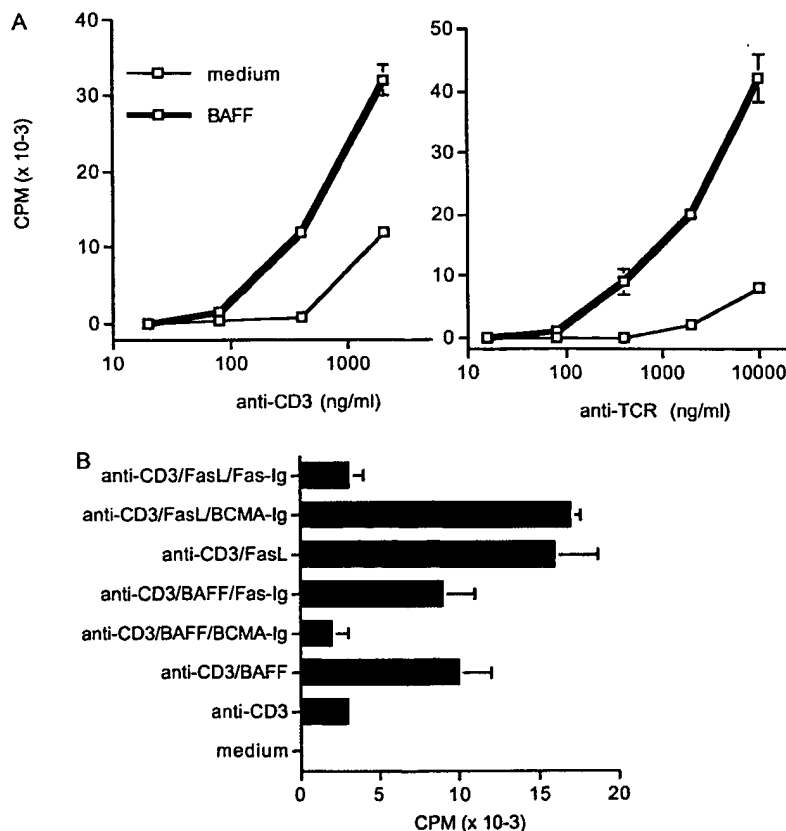
Mingen). Propidium iodide (PI) stainings were performed by incubation of cells in PBS containing 0.5 μ g/ml PI (Sigma-Aldrich) before flow cytometry analysis. Immunostainings were performed, as previously described (13). CFSE and annexin stainings were analyzed on the FL-1 channel, and PI on the FL-3 channel on a FACScan (BD Biosciences, Mountain View, CA) using the CellQuest software.

Results

BAFF induces thymidine incorporation and cytokine secretion of T cells suboptimally stimulated through their TCR

Delivery of signal 1 alone to T cells is known to induce a poor T cell activation. Signal 1 can be reproduced in vitro by using an Ab directed against the TCR-CD3 complex. Fig. 1A shows that stimulation of purified human T cells with increasing concentrations of immobilized Ab against CD3 (left panel) or against the TCR (right panel) resulted in marginal proliferative responses at high Ab concentrations (3 μ g/ml for the anti-CD3, and 3–10 μ g/ml for the anti-TCR). The presence of coimmobilized rBAFF in this assay increased the proliferation to high Ab concentrations and even induced this response at suboptimal concentrations of these Abs. Fig. 1b shows that addition of a soluble form of one BAFF receptor, BCMA (14, 15), BCMA-Ig, in this assay completely inhibited BAFF stimulation. On the contrary, control Fas-Ig did not show any effect. Likewise, BCMA-Ig had no effect on stimulation mediated by an unrelated TNF ligand, FasL (16). In these experiments, addition of a soluble form of the second BAFF receptor, the transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI) (17, 18), also inhibited BAFF stimulation (data not shown). The BAFF stimulation was seen with purified T cells from different donors ($n = 10$). BAFF activity was seen when the recombinant molecule was coated onto plastic surface. Addition of this molecule in a soluble form did not significantly stimulate T cells (data not shown). Immobilization of rBAFF alone in

FIGURE 1. rBAFF induces thymidine incorporation by T cells stimulated with suboptimal doses of anti-TCR/CD3 mAbs. **A**, rBAFF increases thymidine incorporation by T cells. Purified T cells were incubated with increasing concentrations of immobilized Ab against CD3 (OKT3, left panel) or the TCR (BMA 031, right panel) complex in the presence or absence of coimmobilized rBAFF (10 μ g/ml). The proliferative responses were monitored during the last 18 h of a 72-h incubation. The results are expressed as the mean of triplicate cultures \pm SD. **B**, The BAFF stimulation activity is blocked by BCMA-Ig. Proliferative responses were monitored as in **A**. Anti-CD3 was used at 0.3 μ g/ml. Soluble BCMA-Ig and Fas-Ig were added at 50 μ g/ml. The inhibition mediated by BCMA-Ig was observed in three separate experiments.



this experiment did not provide a stimulation by itself (data not shown), reflecting a dependency on TCR/CD3 signaling, and thereby demonstrating that the signal delivered to T cells is a costimulatory signal.

In this T cell stimulation assay, anti-CD28 Abs were used to compare the potency of the BAFF stimulation pathway with the well-described CD28 one. We found donors responding better to the BAFF stimulation, as well as donors responding better to the CD28 stimulation. Fig. 2 shows a comparison of stimulation indexes (SI) obtained with rBAFF or the anti-CD28, mAb 9.3, on T cell proliferation of different donors. Overall, SI obtained with these two reagents were comparable over the range of experiments performed, with a mean of about 30. Similar results were obtained with a second anti-CD28 mAb (CD28.2) (data not shown). These data demonstrate that BAFF provides a potent costimulatory signal to T cells in this assay.

We next assessed CD4⁺ vs CD8⁺ T cells for their responsiveness to BAFF costimulation. As with total T cells, a costimulation in the proliferation of purified CD4⁺ T cells was observed (see Fig. 6), but no reproducible proliferation could be obtained with purified CD8⁺ T cells (data not shown). By studying cytokine secretion of these two T cell subsets, we observed that rBAFF induced TNF- α (left panel) and IFN- γ (right panel) secretion by CD4⁺ as well as CD8⁺ T cells (Fig. 3). The induction was observed when these cells were stimulated with a suboptimal concentration of anti-CD3 mAb. Addition of BCMA-Ig significantly inhibited TNF- α and IFN- γ secretion in this experiment. Taken together, these data indicate that BAFF costimulation induces a response (thymidine incorporation and cytokine secretion) in helper as well as cytotoxic T cells, suboptimally stimulated through their TCR.

BAFF costimulation drives T cell division in an IL-2-dependent manner

To insure that the thymidine incorporation reported above corresponded to cell proliferation, we first looked for IL-2 production and CD25 induction. Fig. 4A shows that significant levels of IL-2 (22 IU/ml) were detected in the supernatant of T cells stimulated for 24 h with the anti-CD3 and rBAFF. Without rBAFF, no IL-2

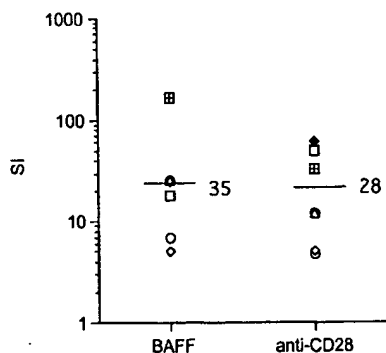


FIGURE 2. SIs on thymidine incorporation by T cells obtained with rBAFF or anti-CD28. T cell proliferation assays with an anti-CD3 in the presence of immobilized BAFF (left) or soluble anti-CD28 (right) were performed as described in the legend to Fig. 1. SI were calculated according to the formula: cpm (T cells stimulated with anti-CD3 and BAFF or anti-CD28)/cpm (T cells stimulated with anti-CD3 alone). Background cpm obtained with T cells in medium alone were negligible. SI shown represent assays wherein the anti-CD3 was suboptimal (<1000 cpm obtained). Each symbol represents one donor ($n = 7$). The mean SI obtained with these seven donors is indicated. The anti-CD28 mAb 9.3 was used at an optimal concentration (100 ng/ml).

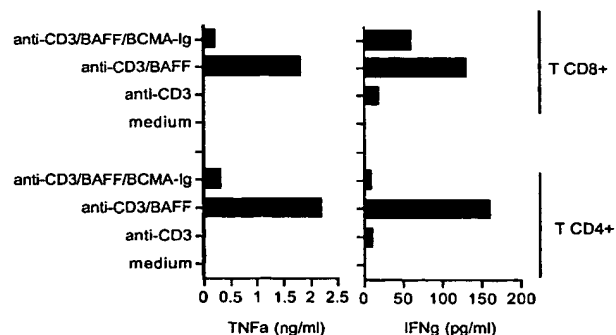


FIGURE 3. rBAFF induces cytokine secretion by CD4⁺ and CD8⁺ T cells stimulated with a suboptimal dose of anti-CD3. Purified CD4⁺ or CD8⁺ T cells were incubated with immobilized rBAFF (10 μ g/ml) and a suboptimal concentration of anti-CD3. For CD4⁺ T cells, the anti-CD3 mAb was used at 1 μ g/ml for both IFN- γ and TNF- α assays. For CD8⁺ T cells, the anti-CD3 mAb was used at 0.3 and 0.1 μ g/ml for TNF- α and IFN- γ assays, respectively. Supernatants were harvested at 72 h for quantification of cytokine secretion in a standard sandwich ELISA. Similar results were obtained in three independent experiments.

was detected. Fig. 4B shows that BAFF costimulation results in a strong increase in CD25 membrane expression. Twenty-four hours after stimulation, 27% of the T cells expressed CD25 in the presence of rBAFF, while only 4.5% expressed this Ag in its absence. This CD25 induction was blocked by the addition of the BAFF antagonist BCMA-Ig. Similar results were also obtained at later time points (48 h, Fig. 4B; 96 h, data not shown). This strongly suggests that, in the presence of BAFF, a higher proportion of T cells expressed a high affinity receptor for IL-2 and could therefore respond to the IL-2 produced. Fig. 4C shows that addition of the anti-CD25 Ab (MAR 93), which blocks IL-2 signaling, almost completely blocked proliferation obtained in the presence of rBAFF. This experiment demonstrates that BAFF-mediated proliferation is IL-2 dependent.

We next studied cell division. T cell division was assessed by CFSE staining and flow cytometry analysis of stimulated T cells. Four days after stimulation, we found that in the presence of BAFF costimulation, 33% of T cells had divided, while only 3.5% had divided in its absence (Fig. 5A). In this experiment, costimulation with an anti-CD28 (mAb 9.3) gave a comparable percentage of dividing T cells (data not shown). Noteworthy, these dividing T cells excluded PI, demonstrating that they were viable stimulated T cells. In fact, we could not observe any significant modulation in the percentage of apoptotic (annexin positive) or dead cells (PI positive) at any time (from 15 h up to 4 days) after anti-CD3 stimulation in the presence of BAFF (Fig. 5B). We do not consider the increase in the percentage of annexin⁺ T cells (9 and 13% in the absence and in the presence of BAFF, respectively) 42 h after activation significant. Indeed, this increase was not confirmed in the second experiment performed, and we did not recover these cells when the PI⁺ fraction was studied at later time points ($n = 4$). These experiments indicate that BAFF costimulation on T cells is sufficient to drive T cell division by inducing factors necessary for T cell growth (IL-2 and CD25).

Naive as well as memory/effector T cells respond to BAFF costimulation

It is known that mature T cells, depending on their prior Ag encounter, do not have the same requirement for activation signal, naive T cells being the subset that is more refractory to activation signals. To assess whether BAFF costimulation is potent on naive

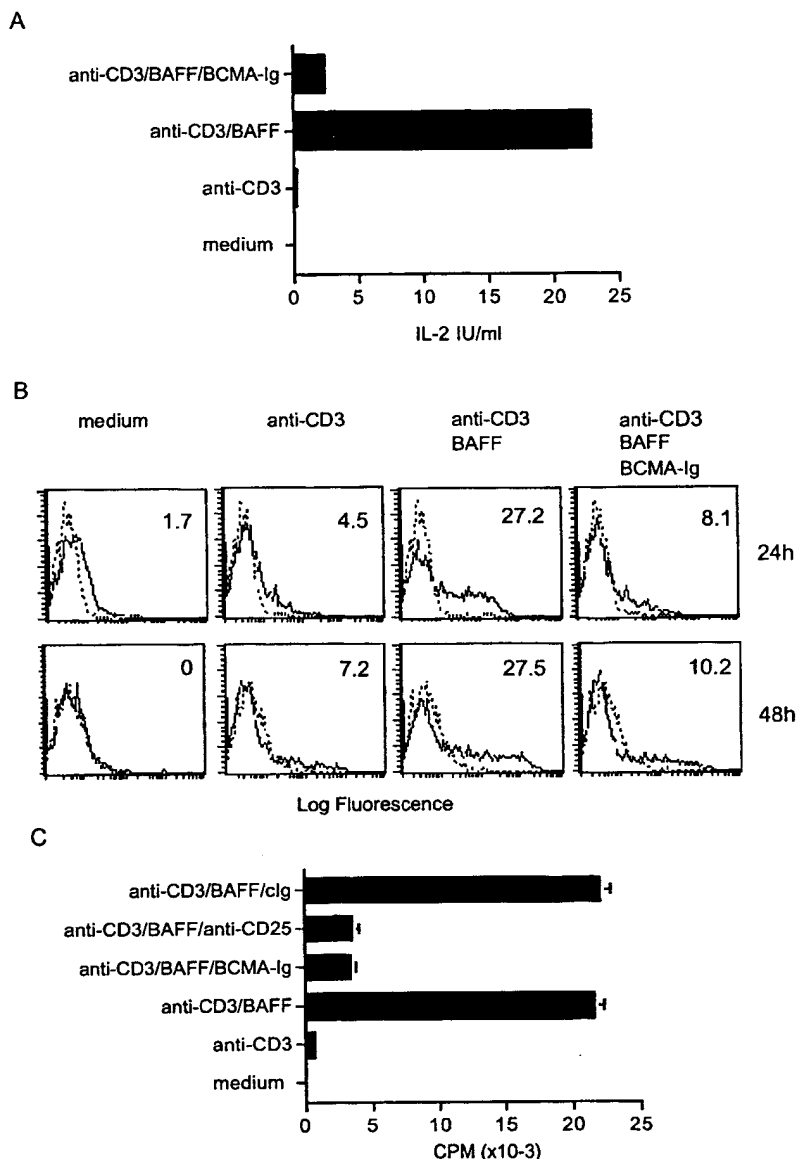


FIGURE 4. BAFF costimulation induces IL-2 secretion and CD25 membrane expression, and drives IL-2-dependent proliferation of T cells. *A*, BAFF stimulation induces IL-2 secretion: T cells were stimulated for 24 h. Supernatants were harvested and assessed for IL-2 in a standard CTLL2 assay. IL-2 concentrations are shown. *B*, BAFF stimulation up-regulates CD25 membrane expression: T cells were stimulated for the indicated time. Cells were harvested, stained with an anti-CD25, and analyzed by flow cytometry. Fluorescence profiles corresponding to anti-CD25 stainings (solid lines) and control stainings (dashed lines) are shown. Percentages of CD25-stained cells are indicated. *C*, BAFF-induced proliferation is IL-2 dependent: The proliferative responses were monitored as in Fig. 1*A*. The anti-CD25 was used at 20 μ g/ml. cIg was an anti-KIR2DL1 used at the same concentration. Anti-CD3 and BAFF were used at 1 and 10 μ g/ml, respectively. BCMA-Ig was used at 50 μ g/ml. The experiments shown are representative of three independent experiments with T cells derived from two different donors.

cells, we further separated CD4⁺ T cells into naive and memory cells based on their expression of CD45RA and CD45RO, respectively. Fig. 6 shows that the BAFF costimulation described above for total T cells was reproduced on memory cells (*middle panel*). In this experiment, even though the proliferation obtained was lower, rBAFF also significantly costimulated the proliferation of naive CD4⁺ T cells (Fig. 6, *upper panel*). Proliferation of total T cells from this donor is shown for comparison (Fig. 6, *lower panel*). Costimulation of naive T cells was reproduced with cells derived from four independent donors (data not shown).

To document the activity of rBAFF on Ag-experienced T cells, we studied whether recently activated T cells could also respond to BAFF costimulation. As recently activated T cells, we used in vitro propagated CD8⁺ T cell clone and bulk populations of T cells activated with PHA. Fig. 7*A* shows that stimulation of the T cell clone in the presence of rBAFF resulted in the induction of IFN- γ secretion, when a suboptimal concentration of an anti-TCR mAb was used. In this experiment, LIGHT, another member of the TNF ligand family (19), was used as a control molecule and gave reproducibly no induction of IFN- γ by this T cell clone. Similarly, induction of IFN- γ was obtained when starved, PHA-activated T

cells were used in this experiment (Fig. 7*B*, *lower panel*). We also assessed induction of type II cytokines (IL-4, IL-5, and IL-13) by these PHA-activated T cells. In addition to IFN- γ induction, rBAFF induced ng/ml levels of IL-5 and IL-13 (Fig. 7*B*, *middle panel*) and weak IL-4 secretion (Fig. 7*C*, *upper panel*). This experiment shows that BAFF costimulation of T cell cytokine secretion is not selective for a type of cytokine. Type I and II cytokines appear to be induced similarly. To rule out any putative polarization signal mediated by BAFF on T cell differentiation, we performed T cell polarization experiments. When total T cells were stimulated with anti-CD3, the percentage of IFN- γ vs IL-4-secreting T cells upon restimulation did not change when BAFF was present during the stimulation phase (data not shown). This was further detailed in an experiment wherein naive T cells were used. The amount of type I (IFN- γ) as well as type II (IL-5 and IL-13) cytokines secreted by these T cells did not change significantly in the presence of rBAFF, even after two cycles of stimulation (data not shown). Altogether, these experiments show that BAFF has a wide costimulatory activity on T cell responses. The costimulation is evident on proliferation as well as cytokine secretion, without

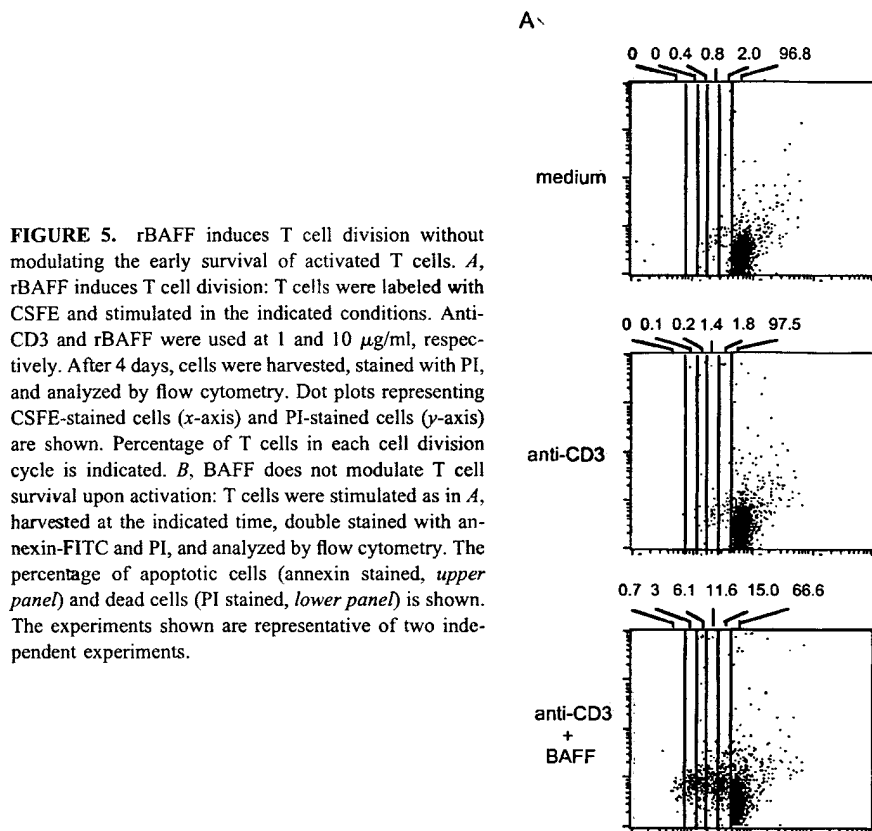


FIGURE 5. rBAFF induces T cell division without modulating the early survival of activated T cells. *A*, rBAFF induces T cell division: T cells were labeled with CFSE and stimulated in the indicated conditions. Anti-CD3 and rBAFF were used at 1 and 10 $\mu\text{g/ml}$, respectively. After 4 days, cells were harvested, stained with PI, and analyzed by flow cytometry. Dot plots representing CFSE-stained cells (x-axis) and PI-stained cells (y-axis) are shown. Percentage of T cells in each cell division cycle is indicated. *B*, BAFF does not modulate T cell survival upon activation: T cells were stimulated as in *A*, harvested at the indicated time, double stained with annexin-FITC and PI, and analyzed by flow cytometry. The percentage of apoptotic cells (annexin stained, upper panel) and dead cells (PI stained, lower panel) is shown. The experiments shown are representative of two independent experiments.

any evident specificity for the type of cytokines induced. Importantly, naive, as well as effector/memory T cells are responsive to this newly identified costimulation pathway.

Discussion

In this study, we report that BAFF, a TNF ligand recently described as an important molecule in the development of B cell

responses, also has an activity on T cell responses. BAFF induced thymidine incorporation and cytokine secretion of T cells in the presence of a suboptimal signal 1. For cytokine secretion, no selectivity in the type of cytokines induced was detected in the presence of BAFF stimulation. IFN- γ as well as IL-5 and IL-13 were all induced in preactivated T cells by BAFF. BAFF activity was observed on mature T cells at all stages of differentiation. BAFF stimulated thymidine incorporation of naive T cells, recently primed T cells, and memory T cells. The wide activity of BAFF on T cell responses is further strengthened by the observation that both CD4 helper and CD8 cytotoxic T cells were found responsive to this costimulation pathway. Importantly, T cell costimulation by BAFF resulted in the production of IL-2 and induction of the high-affinity component of its receptor, CD25, which together drove T cell division. The BAFF costimulation pathway is therefore a complete signal 2, known to be essential for T cell division and expansion.

It is of interest to note that many T cell stimulatory molecules do not induce detectable levels of IL-2 (20). For example, among the different T cell stimulatory molecules from the TNF superfamily, only 4-1BBL has been shown to induce IL-2 production by T cells. In this regard, BAFF costimulation may be comparable with the well-described signal 2 provided by CD28. In our *in vitro* T cell proliferation assays (thymidine incorporation and CFSE staining), we observed a similar potency for BAFF and two different anti-CD28 mAbs. Noteworthy, we observed this BAFF activity in absence of any potent CD28 signaling (purified T cells in the absence of APCs). This indicates that the BAFF costimulation pathway may play a substitutive role, when the CD28 pathway is absent.

BAFF mRNA has been detected constitutively in monocytes, dendritic cells, as well as in T cells with an up-regulation upon

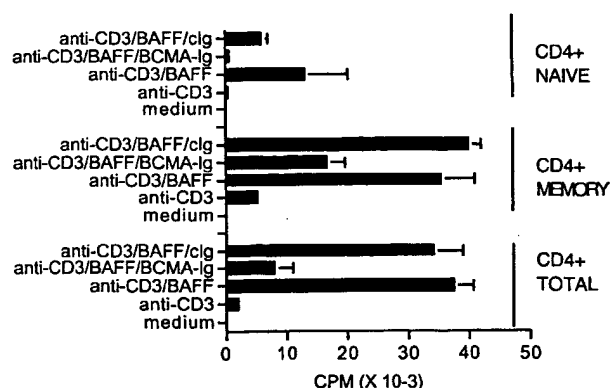


FIGURE 6. rBAFF costimulates the proliferation of naive and memory T cells. Purified T cells were subdivided into naive and memory T cells according to their membrane expression of CD45RA and CD45RO Ags, respectively. These cells were stimulated in the indicated conditions, and proliferation was assessed as in Fig. 1 legend. The anti-CD3 and rBAFF were used at 10 $\mu\text{g/ml}$; BCMA-Ig and the cIg (TNF-2 related apoptosis-inducing ligand-R1-Ig) were used at 50 $\mu\text{g/ml}$. Unfractionated T cells are shown for comparison. A similar result was obtained with naive and memory cells purified from a second independent donor.

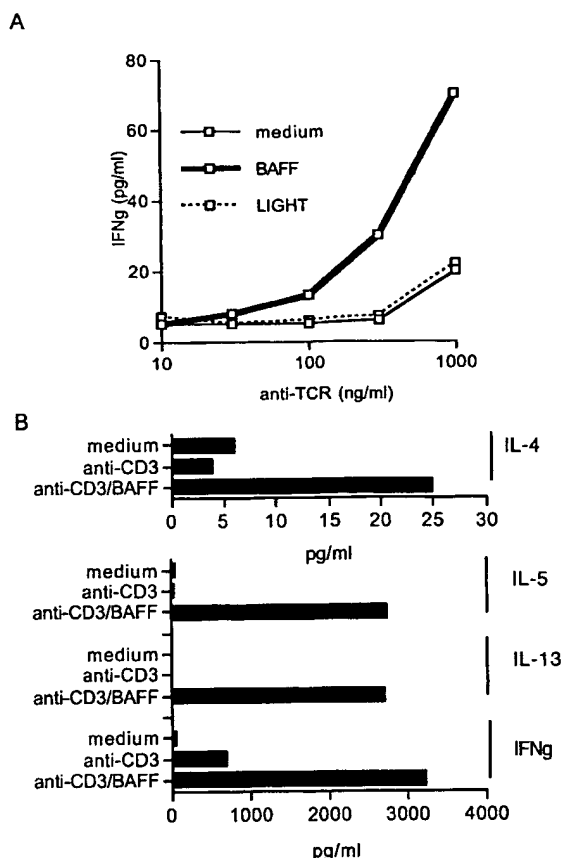


FIGURE 7. rBAFF induces cytokine secretion of recently activated T cells. *A*, BAFF induces IFN- γ in a CD8 $^{+}$ T cell clone: A CD8 $^{+}$ T cell clone was restimulated with an anti-TCR at the indicated concentrations. BAFF and LIGHT were immobilized at 10 μ g/ml. Supernatants were harvested at 48 h and tested for IFN- γ in a standard sandwich ELISA. Similar results were reproduced in a second experiment. *B*, BAFF induces type I and II cytokines in PHA-activated T cells: PHA-activated T cells were restimulated with a suboptimal dose of anti-CD3 (10 ng/ml). Supernatants were harvested at 48 h and tested for the indicated cytokines in standard sandwich ELISAs. This experiment is representative of three independent experiments.

cellular activation in both CD4 $^{+}$ and CD8 $^{+}$ T cell subsets (Ref. 8 and our unpublished data). Such expression pattern appears to be common among stimulatory molecules belonging to the TNF ligand family (e.g., OX40L, 4-1BBL, CD70, and LIGHT). This expression pattern indicates that T cell costimulation mediated by BAFF may be delivered by APCs during APC/T cell interactions. It could not be excluded that BAFF may act also during the T cell expansion phase, in an autocrine fashion, once T cells have detached from APCs.

BAFF is predicted to be expressed as a soluble molecule, due to a furin-like protease site in its ectodomain. This site is effective in transfected epithelial cells, and most of the transfected BAFF is secreted (Ref. 8 and our unpublished observation). On the other hand, a recent observation indicates that BAFF could be detected at the membrane of human monocytes (9), indicating that BAFF cleavage may not be as effective in primary cells. BAFF cleavage is of importance, when one considers BAFF function. Indeed, on B cells, a soluble BAFF trimer is functional for signaling (8, 9, 18), and this signaling is not enhanced by addition of a cross-linking reagent (8). On T cells, our experiments indicate that BAFF needs to be oligomerized (immobilization on plastic surfaces) to signal

into T cells. In vivo, such an oligomerized state for BAFF is likely to be found on a cell surface. These observations suggest a different mechanism of action for BAFF on B cells and on T cells. On B cells, BAFF may act as a cytokine, able to signal into cells even at a distant site from the BAFF-producing cells. The observed B cell dysregulation when BAFF is overexpressed as a transgene under the control of a liver promoter (21) is an argument in favor of this mechanism of action. On the contrary, on T cells, BAFF may signal into cells that are in close contact with the BAFF-producing cells. Such signaling is likely to occur between T cells and APCs in the presence of the Ag.

To date, two BAFF receptors, BCMA and TACI, have been described (14, 15, 17, 22). In addition to its expression on B cells, TACI has been found to be expressed in human activated T cells (23, 24), and rBAFF appears to bind to these activated T cells (18). On the other hand, BCMA appears to be expressed exclusively on B cells (25). Therefore, TACI may be the receptor mediating the BAFF costimulatory activity reported in this work. One should note that BAFF may not be the only TNF ligand signaling into T cells through TACI (or another yet unknown receptor). Indeed, APRIL (a proliferation-inducing ligand, also called TRDL-1) is another member of the TNF ligand family (26, 27) that shares common receptors with BAFF (17, 22, 28). Interestingly, APRIL has also been reported to stimulate T cells in the murine system (22). The involvement of BAFF and APRIL in T cell activation is strengthened by a recent report indicating that a soluble form of TACI inhibited anti-CD3-driven T cell activation in vitro, as well as T cell-mediated pathologies in a collagen-induced arthritis model (24). Further studies are definitely required to dissect the relative contribution of the two related TNF ligands, BAFF and APRIL, on T cell stimulation.

From our study, it can be said that BAFF regulates both B and T cell activation, with an overall enhancement of proliferation and effector responses (Ig secretion for B cells and cytokine secretion for T cells). The enhancement of B cell responses originally described for BAFF (8, 9, 18) is thought to be due to an increase in survival of activated B cells (29). This survival signal has been further confirmed on resting B cells (12). On T cells, we did not detect such increase in T cell survival upon activation, in the presence of BAFF signaling. Enhancement of T cell responses is therefore likely to be due to a BAFF costimulatory activity, important for the development of a full T cell response in the presence of a signal 1. Taken together, these indicate that BAFF may differentially regulate humoral and cellular responses. On humoral responses, BAFF may have a more pronounced quantitative effect, by increasing the number of viable Ag-responding B cells. On cellular responses, the effect of BAFF may be more qualitative, by allowing the development of an optimal response in Ag-responding T cells.

Note added in proof. Recently, a third receptor for BAFF, not shared by APRIL, has been reported (30, 31).

Acknowledgments

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References

- Chambers, C. A., and J. P. Allison. 1997. Co-stimulation in T cell responses. *Curr. Opin. Immunol.* 9:396.
- Locksley, R. M., N. Killeen, and M. J. Lenardo. 2001. The TNF and TNF receptor superfamilies: integrating mammalian biology. *Cell* 104:487.
- Weinberg, A. D., A. T. Vella, and M. Croft. 1998. OX-40: life beyond the effector T cell stage. *Semin. Immunol.* 10:471.
- Vinay, D. S., and B. S. Kwon. 1998. Role of 4-1BB in immune responses. *Semin. Immunol.* 10:481.

5. Lens, S. M., K. Tesselaar, M. H. van Oers, and R. A. van Lier. 1998. Control of lymphocyte function through CD27-CD70 interactions. *Semin. Immunol.* 10:491.
6. Tamada, K., K. Shimozaki, A. I. Chapoval, Y. Zhai, J. Su, S. F. Chen, S. L. Hsieh, S. Nagata, J. Ni, and L. Chen. 2000. LIGHT, a TNF-like molecule, costimulates T cell proliferation and is required for dendritic cell-mediated allogeneic T cell response. *J. Immunol.* 164:4105.
7. Harrop, J. A., M. Reddy, K. Dede, M. Brigham-Burke, S. Lyn, K. B. Tan, C. Silverman, C. Eichman, R. DiPrinzio, J. Spampinato, et al. 1998. Antibodies to TR2 (herpesvirus entry mediator), a new member of the TNF receptor superfamily, block T cell proliferation, expression of activation markers, and production of cytokines. *J. Immunol.* 161:1786.
8. Schneider, P., F. MacKay, V. Steiner, K. Hofmann, J. L. Bodmer, N. Holler, C. Ambrose, P. Lawton, S. Bixler, H. Acha-Orbea, et al. 1999. BAFF, a novel ligand of the tumor necrosis factor family, stimulates B cell growth. *J. Exp. Med.* 189:1747.
9. Moore, P. A., O. Belvedere, A. Orr, K. Pieri, D. W. LaFleur, P. Feng, D. Soppet, M. Charters, R. Gentz, D. Parmelee, et al. 1999. BLYS: member of the tumor necrosis factor family and B lymphocyte stimulator. *Science* 285:260.
10. Shu, H. B., W. H. Hu, and H. Johnson. 1999. TALL-1 is a novel member of the TNF family that is down-regulated by mitogens. *J. Leukocyte Biol.* 65:680.
11. Mukhopadhyay, A., J. Ni, Y. Zhai, G. L. Yu, and B. B. Aggarwal. 1999. Identification and characterization of a novel cytokine, THANK, a TNF homologue that activates apoptosis, nuclear factor- κ B, and c-Jun NH₂-terminal kinase. *J. Biol. Chem.* 274:15978.
12. Batten, M., J. Groom, T. G. Cachero, F. Qian, P. Schneider, J. Tschopp, J. L. Browning, and F. Mackay. 2000. BAFF mediates survival of peripheral immature B lymphocytes. *J. Exp. Med.* 192:1453.
13. Huard, B., P. Prigent, M. Tournier, D. Bruniquel, and F. Triebel. 1995. CD4/major histocompatibility complex class II interaction analyzed with CD4- and lymphocyte activation gene-3 (LAG-3)-Ig fusion proteins. *Eur. J. Immunol.* 25:2718.
14. Shu, H. B., and H. Johnson. 2000. B cell maturation protein is a receptor for the tumor necrosis factor family member TALL-1. *Proc. Natl. Acad. Sci. USA* 97:9156.
15. Thompson, J. S., P. Schneider, S. L. Kalled, L. Wang, E. A. Lefevre, T. G. Cachero, F. MacKay, S. A. Bixler, M. Zafari, Z. Y. Liu, et al. 2000. BAFF binds to the tumor necrosis factor receptor-like molecule B cell maturation antigen and is important for maintaining the peripheral B cell population. *J. Exp. Med.* 192:129.
16. Kennedy, N. J., T. Kataoka, J. Tschopp, and R. C. Budd. 1999. Caspase activation is required for T cell proliferation. *J. Exp. Med.* 190:1891.
17. Marsters, S. A., M. Yan, R. M. Pitti, P. E. Haas, V. M. Dixit, and A. Ashkenazi. 2000. Interaction of the TNF homologues BLYS and APRIL with the TNF receptor homologues BCMA and TACI. *Curr. Biol.* 10:785.
18. Xia, X. Z., J. Treanor, G. Senaldi, S. D. Khare, T. Boone, M. Kelley, L. E. Theill, A. Colombero, I. Solovyev, F. Lee, et al. 2000. TACI is a TRAF-interacting receptor for TALL-1, a tumor necrosis factor family member involved in B cell regulation. *J. Exp. Med.* 192:137.
19. Mauri, D. N., R. Ebner, R. I. Montgomery, K. D. Kochel, T. C. Cheung, G. L. Yu, S. Ruben, M. Murphy, R. J. Eisenberg, G. H. Cohen, et al. 1998. LIGHT, a new member of the TNF superfamily, and lymphotoxin α are ligands for herpesvirus entry mediator. *Immunity* 8:21.
20. Watts, T. H., and M. A. DeBenedette. 1999. T cell co-stimulatory molecules other than CD28. *Curr. Opin. Immunol.* 11:286.
21. Mackay, F., S. A. Woodcock, P. Lawton, C. Ambrose, M. Baetscher, P. Schneider, J. Tschopp, and J. L. Browning. 1999. Mice transgenic for BAFF develop lymphocytic disorders along with autoimmune manifestations. *J. Exp. Med.* 190:1697.
22. Yu, G., T. Boone, J. Delaney, N. Hawkins, M. Kelley, M. Ramakrishnan, S. McCabe, W. R. Qiu, M. Kornuc, X. Z. Xia, et al. 2000. APRIL and TALL-1 and receptors BCMA and TACI: system for regulating humoral immunity. *Nat. Immun.* 1:252.
23. Von Bulow, G. U., and R. J. Bram. 1997. NF-AT activation induced by a CAML-interacting member of the tumor necrosis factor receptor superfamily. *Science* 278:138.
24. Wang, H., S. A. Marsters, T. Baker, B. Chan, W. P. Lee, L. Fu, D. Tumas, M. Yan, V. M. Dixit, A. Ashkenazi, and I. S. Grewal. 2001. TACI-ligand interactions are required for T cell activation and collagen-induced arthritis in mice. *Nat. Immun.* 2:632.
25. Laabi, Y., M. P. Gras, J. C. Brouet, R. Berger, C. J. Larsen, and A. Tsapis. 1994. The BCMA gene, preferentially expressed during B lymphoid maturation, is bidirectionally transcribed. *Nucleic Acids Res.* 22:1147.
26. Kelly, K., E. Manos, G. Jensen, L. Nadauld, and D. A. Jones. 2000. APRIL/TRDL-1, a tumor necrosis factor-like ligand, stimulates cell death. *Cancer Res.* 60:1021.
27. Hahne, M., T. Kataoka, M. Schroter, K. Hofmann, M. Irmeler, J. L. Bodmer, P. Schneider, T. Bornand, N. Holler, L. E. French, et al. 1998. APRIL, a new ligand of the tumor necrosis factor family, stimulates tumor cell growth. *J. Exp. Med.* 188:1185.
28. Rennert, P., P. Schneider, T. G. Cachero, J. Thompson, L. Trabach, S. Hertig, N. Holler, F. Qian, C. Mullen, K. Strauch, et al. 2000. A soluble form of B cell maturation antigen, a receptor for the tumor necrosis factor family member APRIL, inhibits tumor cell growth. *J. Exp. Med.* 192:1677.
29. Do, R. K., E. Hatada, H. Lee, M. R. Tourigny, D. Hilbert, and S. Chen-Kiang. 2000. Attenuation of apoptosis underlies B lymphocyte stimulator enhancement of humoral immune response. *J. Exp. Med.* 192:953.
30. Thompson, J. S., S. A. Bixler, F. Qian, K. Vora, M. L. Scott, T. G. Cachero, C. Hession, P. Schneider, I. D. Sizing, C. Mullen, et al. 2001. BAFF-R, a newly sensitized TNF receptor that specifically interacts with BAFF. *Science* 293:2108.
31. Yan, M., J. R. Brady, B. Chan, W. P. Lee, B. Hsu, S. Harless, M. Cancro, I. S. Grewal, and V. M. Dixit. 2001. Identification of a novel receptor for B lymphocyte stimulator that is mutated in a mouse strain with severe B cell deficiency. *Curr. Biol.* 11:1547.

BAFF/BLyS Receptor 3 Binds the B Cell Survival Factor BAFF Ligand through a Discrete Surface Loop and Promotes Processing of NF- κ B2

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Summary

The TNF-like ligand BAFF/BLyS is a potent survival factor for B cells. It binds three receptors: TACI, BCMA, and BR3. We show that BR3 signaling promotes processing of the transcription factor NF- κ B2/p100 to p52. NF- κ B2/p100 cleavage was abrogated in B cells from A/WySnJ mice possessing a mutant BR3 gene, but not in TACI or BCMA null B cells. Furthermore, wild-type mice injected with BAFF-neutralizing BR3-Fc protein showed reduced basal NF- κ B2 activation. BR3-Fc treatment of NZB/WF1 mice, which develop a fatal lupus-like syndrome, inhibited NF- κ B2 processing and attenuated the disease process. Since inhibiting the BR3-BAFF interaction has therapeutic ramifications, the ligand binding interface of BR3 was investigated and found to reside within a 26 residue core domain. When stabilized within a structured β -hairpin peptide, six of these residues were sufficient to confer binding to BAFF.

Introduction

Survival signals are critical for the proper development and maintenance of the immune system as they regulate the magnitude and duration of the immune response. Many cytokines, including members of the TNF family, such as CD40 ligand, have been shown to function as potent survival factors for specific lymphoid populations (Locksley et al., 2001). BAFF (also known as BLyS, TALL-1, zTNF4, THANK, and TNFS 13B) (Moore et al., 1999; Schneider et al., 1999), a recently defined member of the TNF family, is a homotrimeric type 2 transmembrane protein expressed by macrophages, monocytes, and dendritic cells. BAFF, like other members of the TNF family, also exists in a soluble form following cleavage from the cell surface by Furin-type proteases. It shares most sequence similarity with APRIL, another member of the TNF family, which is expressed by lymphoid cells and at high levels by some tumor cells (Hahne et al., 1998). BAFF is critical for the development

and survival of peripheral B cells; mice lacking BAFF display an almost total loss of follicular and marginal zone B cells (Gross et al., 2001; Schiemann et al., 2001). Transgenic mice overexpressing BAFF develop autoimmune disorders characterized by B cell hyperplasia and autoantibody production including anti-DNA and rheumatoid factor. The animals eventually succumb to an immune complex-mediated, lupus-like nephritis (Gross et al., 2000; Khare et al., 2000; Mackay et al., 1999). Intriguingly, humans suffering from autoimmune syndromes, including systemic lupus erythematosus (SLE), rheumatoid arthritis, and Sjögren's syndrome, where end organ damage is primarily in the kidneys, joints, and salivary/lacrimal glands, respectively, have elevated levels of serum BAFF. Furthermore, BAFF levels correlate with disease severity, consistent with a possible role in the pathogenesis of these disabling maladies (Cheema et al., 2001; Groom et al., 2002; Zhang et al., 2001).

Of the three receptors for BAFF, only BR3 (also known as BAFF-R) is specific; the other two, TACI and BCMA, also bind the related ligand APRIL (Gross et al., 2000; Thompson et al., 2001; Yan et al., 2000, 2001a). The extracellular domain of TACI has a characteristic TNFR-like structure encompassing two cysteine-rich domains (CRDs) that are the hallmark of the TNF receptor family. These approximately 40 residue pseudorepeats have a distinct structure, typically characterized by three intrachain disulfides involving six highly conserved cysteines. BCMA is unusual in that it contains only a single canonical CRD. However, BR3 is even more divergent in that its extracellular domain is composed of only a partial CRD, containing four cysteine residues with spacing distinct from other TNFR modules characterized previously (Bodmer et al., 2002; Naismith and Sprang, 1998). Conventional members of the TNFR family utilize two CRDs for binding ligand; contacts stem primarily from analogous loops from each CRD interacting with two distinct surface patches on the ligand (reviewed in Bodmer et al., 2002). Thus, how high-affinity binding to BAFF is achieved by only a single, or partial, CRD, like that of BR3, is unclear. Recent crystal structures of BAFF reveal a trimeric TNF-like fold with several distinguishing features (Karpusas et al., 2002; Liu et al., 2002; Oren et al., 2002); however, no structure of any of the BAFF receptors has been described.

Characterization of naturally occurring mutations and knockout mice have revealed components of the B cell survival pathway potentially engaged by BAFF. Since A/WySnJ mice that possess a mutant BR3 display a profound lack of B cells, akin to BAFF null mice, it has been hypothesized that BR3 must engage a B cell survival pathway (Gross et al., 2001; Schiemann et al., 2001; Thompson et al., 2001; Yan et al., 2001a). Further, the NF- κ B pathway is an attractive candidate for activation by BR3 because certain components are required for B cell survival and maintenance (Caamano et al., 1998; Franzoso et al., 1998). For example, IKK α , one of two catalytic subunits within the I κ B kinase complex, is required for B cell maturation, formation of secondary

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Fig. 1a

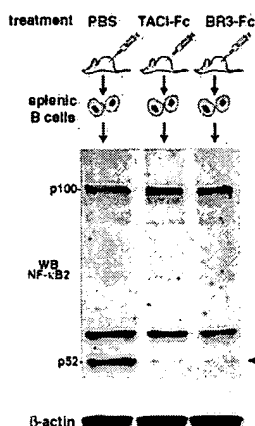


Fig. 1b

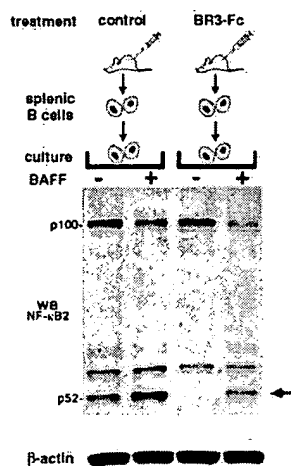


Fig. 1c

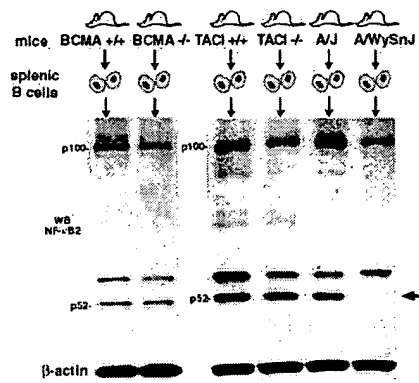


Figure 1. Involvement of Endogenous BAFF/BR3 in Basal Processing of NF- κ B2 In Vivo

(A) Five C57BL/6 wild-type mice were injected intraperitoneally with PBS, 100 μ g TACI-Fc, or 100 μ g BR3-Fc daily for 5 days. 10 μ g pooled splenic B cell lysate from each group was subjected to SDS-PAGE followed by Western blot analysis with anti-NF- κ B2 or anti- β actin. (B) Splenic B cells from PBS- or BR3-Fc-treated mice as described above were cultured with or without 1 μ g/ml recombinant BAFF for 24 hr. Thereafter, 10 μ g cultured B cell lysates were subjected to Western blot analysis with anti-NF- κ B2 or anti- β actin. (C) B cells were purified from pooled spleens of C57BL/6 (control), BCMA^{-/-} (Xu and Lam, 2001), TACI^{-/-} (Yan et al., 2001b), A/J, and A/WySnJ mice. 10 μ g cell lysates were subjected to Western blot analysis with anti-NF- κ B2 or anti- β actin.

lymphoid organs, and the inducible processing of the latent transcription factor NF- κ B2 (p100) to the active p52 NF- κ B subunit (Kaisho et al., 2001; Senftleben et al., 2001). Upon ectopic expression, an IKK α -interacting kinase, NIK, is also capable of triggering NF- κ B2/p100 processing (Xiao et al., 2001). Indeed, processing by ectopic NIK is inhibited in IKK α -null lymphoid cells (Senftleben et al., 2001), consistent with IKK α functioning downstream of NIK. In vitro studies suggest that IKK α can directly phosphorylate NF- κ B2/p100, leading to ubiquitin-dependent generation of p52 (Senftleben et al., 2001).

The only known activator of the NIK/IKK α /NF- κ B2 pathway is the receptor for lymphotoxin- β (LT β R) (Yin et al., 2001). Agonistic LT β R antibodies induce NF- κ B2/p100 processing in a NIK- and IKK α -dependent manner (Ghosh and Karin, 2002). LT β R, however, is expressed on stromal cells and not on B lymphocytes, so the B cell ligand/receptor responsible for engaging the NIK/IKK α /NF- κ B2 pathway has been enigmatic. BAFF (Moore et al., 1999; Schneider et al., 1999), as a potent survival factor for B cells, is an attractive candidate along with its receptors TACI, BCMA, and BR3/BAFF-R (Gross et al., 2000, 2001; Schiemann et al., 2001; Thompson et al., 2001; Yan et al., 2000, 2001a). Of the three receptors, BR3 is most likely to signal B cell survival, as the BR3/BAFF-R mutant mouse strain A/WySnJ has defects in B cell maturation and lymphoid organ architecture similar to that seen in NIK (Yin et al., 2001)-, IKK α (Kaisho et al., 2001; Senftleben et al., 2001)- and NF- κ B2-deficient mice (Caamano et al., 1998; Franzoso et al., 1998). In contrast, BCMA null mice have no discernable phenotype (Schiemann et al., 2001; Xu and Lam, 2001), and TACI null mice possess hyperresponsive B cells, splenomegaly, and increased Ig levels (Yan et al., 2001b), consistent with TACI functioning as an inhibitory receptor.

Herein, we show that the BAFF-BR3 interaction does indeed promote processing of NF- κ B2/p100, both physiologically and in a murine model of lupus. Additionally, we demonstrate that BR3 binds BAFF through a discrete surface loop; structural mimics of this loop could be used to develop inhibitors to treat autoimmune disorders.

Results and Discussion

BAFF/BR3 Signaling Promotes Processing of NF- κ B2

Initially, we determined the impact of BAFF on NF- κ B2/p100 processing in the whole animal. As reported previously (Senftleben et al., 2001; Yamada et al., 2000), splenic B cells from untreated, naive mice display basal activation of the NIK/IKK α /NF- κ B2 pathway as evidenced by the presence of p52. This signaling presumably results from the presence of an endogenous activator, possibly BAFF, in the splenic microenvironment. To test this possibility, C57BL/6 mice were injected with either purified recombinant TACI-Fc, which neutralizes both BAFF and APRIL, or BR3-Fc, which specifically binds BAFF (Gross et al., 2000; Thompson et al., 2001; Yan et al., 2000, 2001a). In either case, generation of p52 was diminished substantially, implying that endogenous BAFF is required for normal basal processing of NF- κ B2/p100 in splenic B cells in vivo (Figure 1A). Nevertheless, BAFF may effect cleavage of NF- κ B2/p100 indirectly by inducing other cytokines that activate NF- κ B2/p100. To eliminate any contribution from accessory cells, splenic B cells purified from mice treated with BR3-Fc (to reduce basal p52 generation) were cultured in the presence of recombinant BAFF. Generation of p52 was augmented significantly in the presence of BAFF, consistent with

Fig. 2a

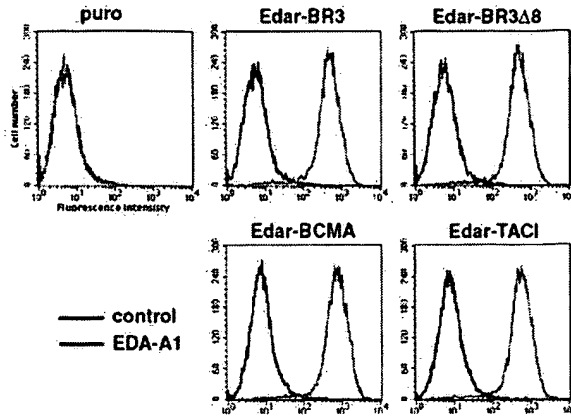


Figure 2. BR3 Activation Is Sufficient for NF- κ B2 Processing (p100 to p52)

(A) WEHI 231 murine B lymphoma cells were infected retrovirally with vector alone (puro) or encoding Edar-BR3, Edar-BR3 Δ 8, Edar-BCMA, and Edar-TACI fusion constructs. The transfectants were stained with FLAG-EDA-A1 followed by anti-FLAG and PE-conjugated anti-rabbit. After washing, cells were analyzed on a FACScan (Becton Dickinson) and data processed using the CELLQuest program (Becton Dickinson).

(B) WEHI 231 transfectants were cultured with 1 μ g/ml FLAG-EDA-A1 for 24 hr. 10 μ g cell lysates of individual transfectants were subjected to Western blot analysis with anti-NF- κ B2 or anti- β actin.

(C) WEHI 231 transfectants were cultured with 1 μ g/ml FLAG-EDA-A1 for indicated periods. Cell lysates (10 μ g) were subjected to Western blot analysis with anti-phospho-I κ B- α and anti- β actin as a loading control.

Fig. 2b

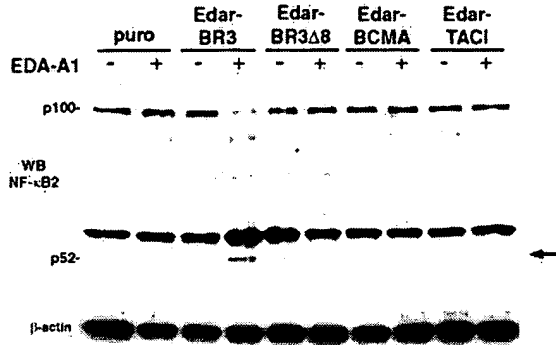
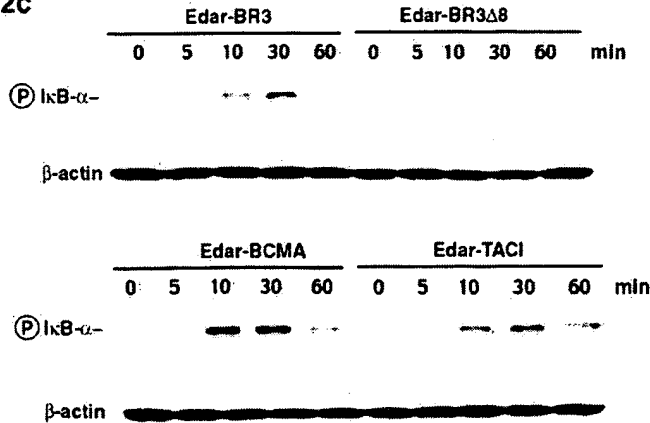


Fig. 2c



BAFF acting directly on B cells to promote NF- κ B2 processing (Figure 1B, right 2 lanes). BAFF also increased, to a lesser extent, the amount of p52 in splenic B cells from untreated wild-type mice (Figure 1B, left 2 lanes), suggesting that endogenous levels of BAFF are not saturating.

Since B cells express all three BAFF receptors (Gross

et al., 2000; Schiemann et al., 2001; Thompson et al., 2001; Yan et al., 2000, 2001a), we determined the contribution of each to NF- κ B2 processing. Similar levels of processed p52 were found in B cells from control C57BL/6, BCMA-, and TACI-deficient mice (Figure 1C). In sharp contrast, no p52 was detected in B cells from the BR3 mutant A/WySnJ strain. These results con-

Fig. 3a

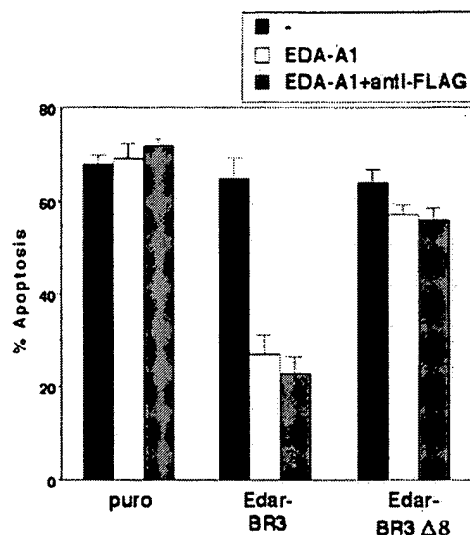


Fig. 3b

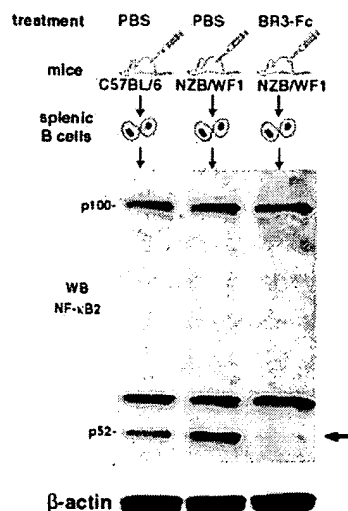


Figure 3. BR3 Crosslinking Can Rescue Anti-IgM-Induced Apoptosis

(A) WEHI 231 transfectants were cultured with or without 1 μ g/ml FLAG-EDA-A1 \pm 5 μ g/ml anti-FLAG Ab for 24 hr followed by coculture with 10 μ g/ml anti-IgM for 48 hr. Cells were then stained with FITC-AnnexinV (Becton Dickinson) and analyzed on a FACScan (Becton Dickinson). Data represent the mean \pm SD of triplicate samples.

(B) Five 24-week-old NZB/WF1 mice were injected intraperitoneally with PBS or 100 μ g BR3-Fc fusion protein three times a week for 5 weeks. 10 μ g cell lysates from pooled splenic B cells of 54-week-old mice were subjected to Western blot analysis with anti-NF- κ B2 or anti- β actin. Age-matched C57BL/6 mice were used as control.

firmed a critical role for BR3 in BAFF-induced processing of NF- κ B2 in B cells.

To extend these *in vivo* observations, we determined the ability of individual BAFF receptors to induce NF- κ B2 processing *in vitro*. A receptor chimera system was used to stimulate each receptor specifically and to avoid activating endogenous BAFF receptors. Thus, the extracellular ligand binding domains of BCMA, TACI, and BR3 were replaced by the extracellular domain of the ectodysplasin receptor (Edar), a TNF receptor family member expressed in developing skin but not in lymphoid or hematopoietic tissues. Retroviral vectors were used to express Edar-BCMA, Edar-TACI, and Edar-BR3 in the murine B cell line WEHI 231. Expression of the chimeric receptors was confirmed by flow cytometric analysis of cells surface stained with the cognate ligand for Edar, EDA-A1 (Figure 2A). Transfectants were cultured in the presence of EDA-A1 to crosslink and activate the chimeric receptors and then examined for the presence of p52. As shown in Figure 2B, control puromycin-resistant cells only expressed unprocessed NF- κ B2/p100. In contrast, p52 was induced in Edar-BR3 transfectants treated with EDA-A1. In keeping with the *in vivo* data (Figure 1C), p52 was not generated in either Edar-BCMA or Edar-TACI transfectants, despite prolonged culture with EDA-A1 (Figure 2B). Taken together, these data suggest that BR3 alone can induce proteolytic maturation of NF- κ B2 to p52. To mimic the insertional mutation that disrupts the eight C-terminal amino acids of BR3 in A/WySnJ mice (Yan et al., 2001a), we

generated an Edar receptor chimera lacking these eight residues (Edar-BR3Δ8). This mutant receptor chimera failed to initiate p100 processing following EDA-A1 treatment (Figure 2B), suggesting that the BR3 C terminus is critical for NF- κ B2 activation.

Most TNFR members can also activate the classical NF- κ B pathway (Ghosh and Karin, 2002). In this pathway, IKK β phosphorylates I κ B, resulting in ubiquitin-dependent degradation of I κ B and subsequent translocation of the cytoplasmic p50/p65 transcriptional complex to the nucleus. To determine if any of the BAFF receptors engaged this pathway, we examined the ability of individual BAFF receptors to induce phosphorylation of I κ B in WEHI231 transfectants. As shown in Figure 2C, all three BAFF receptors induced phospho-I κ B (Ser32/36) within 10 min of activation. BR3Δ8 was unable to induce I κ B phosphorylation, suggesting that the C-terminal eight amino acid residues are important for engaging both the classical NF- κ B and the NF- κ B2 pathways. While these data indicate that all three BAFF receptors can activate the classical NF- κ B pathway, the physiological significance of this is still unclear.

BR3 Signaling Protects WEHI 231 Cells from Anti-IgM-Induced Apoptosis

Potentially harmful, autoreactive B cells are likely kept in check by multiple mechanisms including deletion (Goodnow, 1992; Hertz and Nemazee, 1998; Klinman, 1996), but inappropriately high levels of a B cell survival factor, such as BAFF, may overcome such regulatory

Fig. 4a

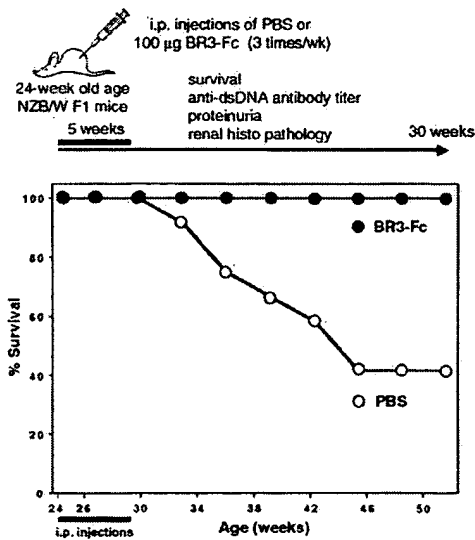


Fig. 4c

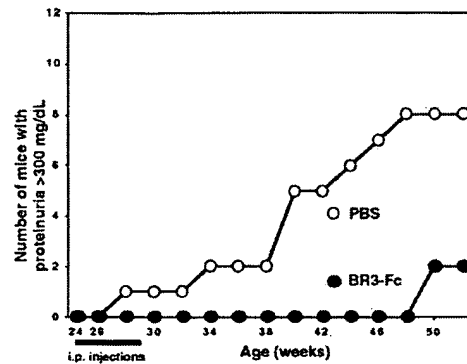


Fig. 4b

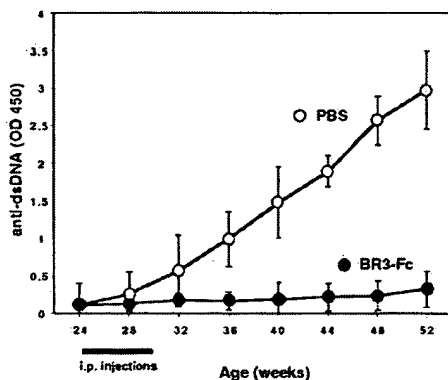


Fig. 4d

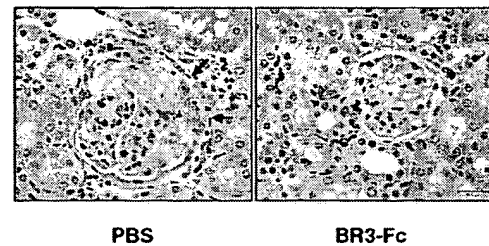


Figure 4. BR3-Fc Blocks Development of Lupus Disease in NZB/WF1 Mice

(A) 24-week-old female NZB/WF1 mice (12 mice per group) were injected intraperitoneally with PBS (open circles) or 100 µg BR3-Fc (filled circles) three times a week for 5 weeks. Mice were checked three times a week for morbidity. The number of mice surviving in each treatment group is shown.

(B) Serum levels of circulating anti-dsDNA antibodies were checked monthly and are shown as OD₄₅₀ values from an ELISA.

(C) Proteinuria was checked monthly and the percentage of mice in each group with levels greater than 300 mg/dL is shown.

(D) 54-week-old NZB/WF1 kidneys from both treatment groups. Arrows indicate fibrinoid necrosis and sclerosis prominent in affected glomeruli accompanied by occasional crescent formation within the PBS-treated mice (left panel) compared to minimal glomerular changes in BR3-Fc-treated animals (right panel).

mechanisms. Indeed, elevated BAFF serum levels have been reported for patients with SLE (Zhang et al., 2001) and Sjögren's syndrome (Groom et al., 2002), although a causal link is yet to be established. An *in vitro* model for B cell negative selection is the induction of apoptosis in WEHI 231 cells by crosslinking anti-IgM antibody (Benhamou et al., 1990; Hasbold and Klaus, 1990). We found that prior engagement of Edar-BR3 with either FLAG-EDA-A1 alone or FLAG-tagged ligand hyper-crosslinked with anti-FLAG antibody caused a marked reduction in anti-IgM-induced apoptosis. In contrast, crosslinking Edar-BR3Δ8 failed to rescue the anti-IgM stimulated cells (Figure 3A). Thus, the C terminus of BR3 is critical for p52 generation, phospho-I κ B induction, and the pro-survival role of BR3 in B cells.

Blockade of BAFF Signaling in NZB/WF1 Mice Abrogates p52 Generation and the Development of Spontaneous Lupus-like Disease

Given that BR3 signaling can protect WEHI 231 cells from apoptosis induced by engagement of the B cell receptor, inappropriate activation of the BAFF-BR3 axis might contribute to the emergence of autoreactive B cell clones that play a pathogenic role in autoimmune disease. To investigate this possibility, we studied NZB/WF1 mice that develop spontaneous, fatal lupus-like disease. At 22–24 weeks of age, NZB/WF1 mice exhibited a slight but reproducible increase in the level of p52 in their B cells when compared to C57BL/6 mice (Figure 3B). This finding is consistent with the elevated levels of BAFF in NZB/WF1 mice (Gross et al., 2000). To assess

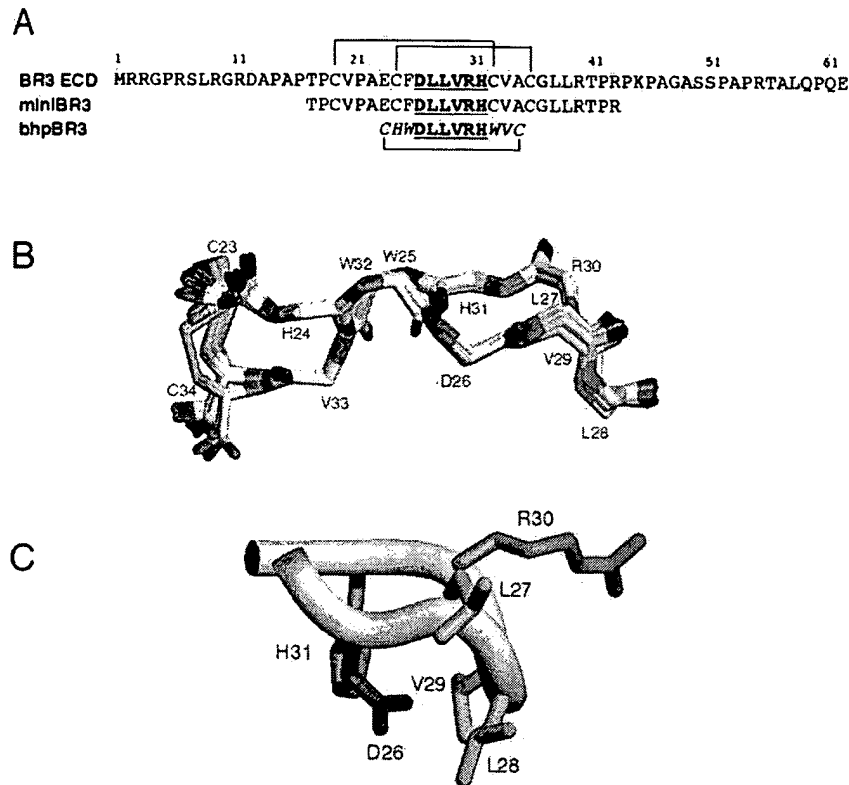


Figure 5. Sequences of BR3 Variants and Structure of bhpBR3

(A) Amino acid sequences of BR3 variants used in this study.

(B and C) Three-dimensional structure of bhpBR3 determined by NMR spectroscopy. The backbone atoms of 20 models are shown superposed with residue labels positioned in the direction of the side chain (B); one representative structure highlighting the BR3 turn residues (C) in the same orientation as in B.

directly the contribution of BAFF to disease progression in NZB/WF1, we treated 24-week-old female animals (12 per group) with a short 5 week course of BR3-Fc. This treatment dramatically attenuated the lupus-like disease for the remaining 30 week length of the study: B cell p52 levels were markedly reduced (Figure 3B), there was a 100% survival rate (Figure 4A), and the mice possessed fewer anti-double-stranded (ds) DNA antibodies (Figure 4B). Renal damage was also less severe as evidenced by diminished proteinuria (Figure 4C) and only mild glomerular changes on renal histology (Figure 4D). Previous studies have shown that treatment with TACI-Fc, which binds both BAFF and APRIL, also suppresses proteinuria but does not block the generation of anti-dsDNA antibodies (Gross et al., 2000). It is not clear why BR3-Fc, which exclusively binds BAFF (Thompson et al., 2001; Yan et al., 2001a), should so potently suppress autoantibody titers, but it may relate to pharmacokinetic differences or a greater potency in neutralizing bioavailable BAFF.

To assess the effect of initial BR3-Fc treatment on long-term leukocyte counts, peripheral blood was analyzed at the time of sacrifice. Leukocyte counts including B cell numbers were within normal limits at the end of the study. Thus, the initial BR3-Fc treatment may have eliminated or silenced B cell populations that play a

pathogenic role in the development of this autoimmune disorder. In contrast, control-treated animals rapidly developed anti-dsDNA autoantibodies (Figure 4B) and succumbed to diffuse membranoproliferative glomerulonephritis characterized by mesangial proliferation and inflammatory cell infiltrates. Fibrinoid necrosis and sclerosis were prominent in affected glomeruli accompanied by occasional crescent formation (Figure 4D). In sum, the data suggest that preventing the BAFF-BR3 interaction may represent a viable therapeutic treatment for lupus.

The BAFF Binding Site within BR3 Is Contained within a 26 Residue Core Domain

As a first step toward developing agents to disrupt the BAFF-BR3 interaction, an NMR analysis of the extracellular ligand binding domain of BR3 was performed. Intriguingly, only the central one-third of the protein adopts a stable structure in solution; this core is stabilized by two disulfide bonds connecting Cys19/Cys32 and Cys24/Cys35 (M.A.S., J.Y., and W.J. Fairbrother, unpublished data). Consequently, a 26 residue miniBR3 peptide was synthesized, incorporating the 1:3, 2:4 disulfide-bonding pattern, and characterized structurally (Figure 5A). Indeed, NMR spectra of miniBR3 indicated that this peptide adopts essentially the same structure

Fig. 6a

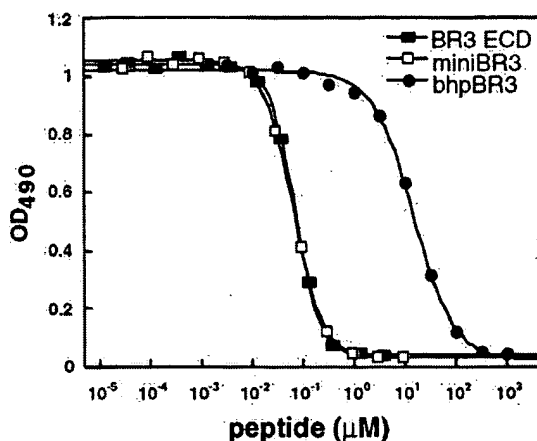


Fig. 6b

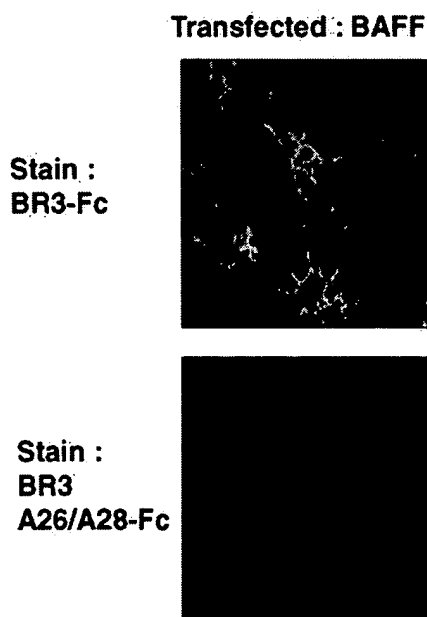


Fig. 6c

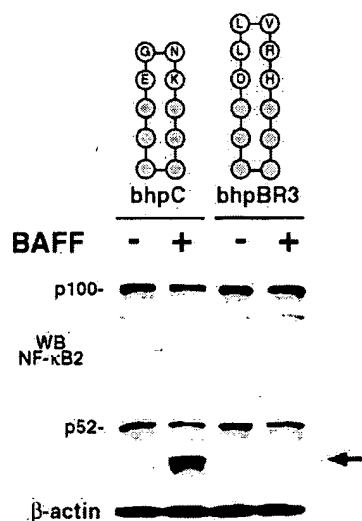


Figure 6. Binding of BR3 Variants to BAFF

(A) Competitive displacement of biotinylated miniBR3 measured by ELISA (see Experimental Procedures). Data are shown for BR3 extracellular domain (filled squares), miniBR3 (open squares), and bhpBR3 (filled circles). IC_{50} values from the fitted curves are 70 nM, 65 nM, and 15 μ M, respectively.

(B) COS 7 cells transfected with BAFF were stained with BR3-Fc or BR3 A26/A28-Fc followed by Cy3-labeled anti-human IgG Fc.

(C) Splenic B cells from BR3-Fc-treated mice were cultured for 24 hr in the presence or absence of recombinant BAFF (2 nM), which had been preincubated for 30 min with bhpBR3 (60 μ M) or a control hairpin peptide composed of an unrelated turn sequence in the same bhp scaffold (bhpC, 60 μ M). Thereafter, B cell lysates (10 μ g) were subjected to Western blot analysis with anti-NF- κ B2 or anti- β actin.

as in the context of the full-length protein (not shown). Importantly, miniBR3 also binds with the same affinity as full-length BR3 to BAFF (\sim 70 nM IC_{50} ; Figure 6A).

Given that high-affinity BAFF binding was contained within a 26 residue core, we attempted to further delin-

eat the BAFF binding portion of BR3. In crystal structures of other TNF-like ligand/receptor complexes, a receptor loop analogous to BR3 residues ²⁶DLLVRH³¹ is involved in forming direct contacts with the ligand (Bodmer et al., 2002, and references therein). Intrigu-

ingly, all three BAFF receptors share the short motif "DxL" at the beginning of this loop (see below). The importance of the conserved motif for BAFF binding was tested by site-directed mutagenesis. As shown in Figure 6B, replacement of the two invariant loop residues of BR3-Fc (D26 and L28) with alanine resulted in complete loss of binding to BAFF, confirming the importance of the loop in BAFF recognition.

In the context of both full-length and miniBR3, NMR analysis suggested that the critical loop sequence ²⁶DLLVRH³¹ presents a type I β turn centered at L28/V29 with potential for backbone hydrogen-bonding between Asp26 and His31. Thus, in order to test whether this loop might be sufficient for binding, we synthesized a 12 residue peptide in which the six residues from BR3 were embedded within a disulfide-bonded β hairpin (bhp) scaffold (Figure 5A). Previously, we have shown that the strong strand-strand interactions in these scaffolds can structure a variety of β turns (Cochran et al., 2001; S.J. Russell, T. Blandl, N.J. Skelton, and A.G.C., unpublished data; Russell and Cochran, 2000).

The peptide bhpBR3 adopts a remarkably stable conformation in solution as indicated by a high degree of chemical shift dispersion, extreme values for many of the backbone and side chain coupling constants, and a large number of long-range NOEs present in its NMR spectra (see supplemental table S1 at <http://www.immunity.com/cgi/content/full/17/4/515/DC1>). The three-dimensional structure of bhpBR3 consists of a β hairpin in which the BR3 turn sequence adopts the type I β turn structure, as expected, with Arg30 adopting a positive ϕ angle and the side chains of the invariant Asp26 and Leu28, as well as those of Val29 and His31, projecting on one face of the β turn (Figures 5B and 5C). If BR3 binds BAFF using interactions homologous to those observed for TNFR and DR5 (Hymowitz et al., 1999), then one would expect this face (the "bottom" face of the turn shown in Figure 5C) to contact BAFF.

Because bhpBR3 structurally mimics the BR3 turn, we tested whether it could compete with miniBR3 for binding to BAFF (Figure 6A). Remarkably, the 12 residue peptide blocked binding of the larger core domain ($IC_{50} = 15 \mu M$), indicating that the critical binding determinants do indeed reside in the six residue loop shown in Figure 5C. Finally, we tested whether bhpBR3 could function in a bioassay: bhpBR3, but not a control hairpin peptide, blocked BAFF-mediated NF- κ B2/p52 induction in primary B cells (Figure 6C).

Our finding that a β turn structure from BR3 has significant affinity for BAFF has implications for recognition of BAFF by its other receptors. TACI and BCMA share homologous sequences in this loop region that would be expected to adopt a similar turn conformation to that in BR3 (D₁LLHA, D₁LLGT, and D₁LLRD, for BCMA and CRDs 1 and 2 of TACI, respectively). Therefore, the interactions of this turn with ligand will likely be a conserved feature of all BAFF/receptor complexes. The surprising identification of such a focused recognition epitope will provide the framework for developing small-molecule peptidomimetic inhibitors of the BAFF-BR3 interaction; these inhibitors may have therapeutic potential in the treatment of autoimmune diseases such as lupus.

Experimental Procedures

Isolation and Culture of B Cells

Splenic B cells were isolated using MACS beads (Miltenyi). Recovered cells were >96% B220⁺IgM⁺ B cells. In some experiments, freshly isolated B cells were further cultured with 1 μ g/ml recombinant BAFF (Yan et al., 2001a) for 24 hr.

Western Blot Analysis

Cell lysis and immunoblotting were performed as described (Humke et al., 2000). Anti-NF- κ B2 (Santa Cruz) was used to detect p100 and p52. Phospho-I κ B- α -specific antibody (Ser32/36, Cell Signaling) was used to detect phospho-I κ B- α .

Retroviral Construction and Infection

Extracellular domains of BCMA (1-42), TACI (1-114), and BR3 (1-55) were replaced by Edar (1-182) by recombinant PCR. Resulting cDNAs were subcloned into a puromycin-selectable retrovirus vector. Production and infection of retrovirus were performed as described elsewhere (Humke et al., 2000). WEHI 231 cells stably expressing chimeric receptors were selected using 1 μ g/ml puromycin.

NZB/WF1 Lupus Mice Studies

Female NZB/WF1 mice were purchased from Jackson. Proteinuria levels were monitored by Uristix (Ames). Anti-dsDNA antibody titers in the serum were measured using poly L-lysine/poly dAdT (Sigma) coated plates and detected with HRP-conjugated goat anti-mouse IgG antibody. In some experiments, kidneys from 54-week old NZB/WF1 mice were fixed in 10% neutral-buffered formalin and embedded in paraffin. Three micron thick sections stained with hematoxylin and eosin were examined by light microscopy.

Protein Production

The extracellular domain of BR3 was subcloned into the pET32a expression vector (Novagen), creating a fusion with an N-terminal thioredoxin (TRX)-His-tag followed by an enterokinase protease site. *E. coli* BL21(DE3) cells (Novagen) were grown at 30°C and protein expression was induced with IPTG. TRX-BR3 was purified over an Ni-NTA column (Qiagen), eluted with an imidazole gradient, and cleaved with enterokinase (Novagen). BR3 was then purified over an S-Sepharose column, refolded overnight in PBS (pH 7.8), in the presence of 3 mM oxidized and 1 mM reduced glutathione, dialyzed against PBS, repurified over a MonoS column, concentrated, and dialyzed into PBS.

Peptide Synthesis

MiniBR3 and bhpBR3 were synthesized as C-terminal amides on a Pioneer peptide synthesizer (PE Biosystems) using standard Fmoc chemistry. For miniBR3, the side chain thiols of cysteines 19 and 32 were protected as the trifluoroacetic acid (TFA)-stable acetamidomethyl (Acm) derivatives. Peptides were cleaved from resin by treatment with 5% triisopropyl silane in TFA for 1.5-4 hr at room temperature. After removal of TFA by rotary evaporation, peptides were precipitated by addition of ethyl ether, then purified by reversed-phase HPLC (acetonitrile/H₂O/0.1% TFA). Peptide identity was confirmed by electrospray mass spectrometry. BhpBR3 was converted to the cyclic disulfide by dropwise addition of a saturated solution of I₂ (in acetic acid) to HPLC fractions. After lyophilization, the oxidized peptide was purified by HPLC. HPLC fractions containing reduced miniBR3 were adjusted to a pH of ~9 with NH₄OH; the disulfide between cysteines 24 and 35 was then formed by addition of a small excess of K₂Fe(CN)₆, and the oxidized peptide purified by HPLC. Acm groups were removed (with concomitant formation of the second disulfide) by treatment of the HPLC eluate with a small excess of I₂ over ~4 hr. The progress of the oxidation was monitored by analytical HPLC, and the final product was again purified by HPLC. MiniBR3 was amino-terminally biotinylated while on resin, then cleaved and purified exactly as described above for the unmodified peptide.

NMR Spectroscopy

Two-dimensional (2D) NMR experiments were acquired and analyzed as described (Starovasnik et al., 1996) using a Bruker DRX-

600 spectrometer at 293K on a sample containing 2.9 mM bhpBR3 (pH 4.5), with 0.1 mM DSS as a chemical shift reference. Distance restraints were derived from 2D NOESY spectra (τ_m 250 ms); dihedral angle restraints were derived from analysis of a 2D DQF-COSY spectrum acquired in 92% H₂O/8% D₂O and a 2D COSY-35 spectrum acquired on a sample dissolved in 100% D₂O. Complete resonance assignments and coupling constant values are included in the supplemental data.

The three-dimensional structure of bhpBR3 was calculated based on 119 NOE-derived (including 46 long-range) distance restraints and 16 dihedral angle restraints. 100 initial structures were calculated using DGIL; 80 of these were further refined by restrained molecular dynamics using DISCOVER as described (Starovasnik et al., 1996). Twenty structures having the lowest restraint violation energy represent the solution conformation of bhpBR3. The model with the lowest rms deviation (RMSD) to the average coordinates of the ensemble was chosen as the representative structure (model 1 in the PDB file). The final ensemble of twenty models satisfies the input data well, having no distance or dihedral angle restraint violations greater than 0.1 Å or 1°, respectively. The structures are well defined, with an average backbone RMSD to the mean coordinates of 0.24 ± 0.06 Å, and have good covalent geometry as judged by PROCHECK (86% of the residues in the most favored, 10% in the allowed, and 4% in the generously allowed regions of $\phi\psi$ space) (Laskowski et al., 1993). The structure of bhpBR3 will be available from the RCSB Protein Data Bank (ID code 1MPV).

Competitive Displacement ELISA

Nunc Maxisorp 96-well plates were coated overnight at 4°C with 100 μ l of a 2 μ g/ml solution of BAFF in carbonate buffer (pH 9.6). The plate was washed with PBS and blocked with 1% skim milk in PBS. Serial dilutions of BR3 variants were prepared in PBS/0.05% Tween 20 containing 3 ng/ml biotinylated miniBR3. After washing with PBS/Tween, 100 μ l/well of each dilution was transferred and incubated for 1 hr at room temperature. The plate was washed with PBS/Tween and incubated for 15 min with 100 μ l/well of 0.1 U/ml Streptavidin-POD (Boehringer Mannheim) in PBS/Tween. After washing with PBS/Tween followed by PBS, the plate was incubated for 5 min with 100 μ l PBS substrate solution containing 0.8 mg/ml OPD (Sigma) and 0.01% H₂O₂. The reaction was quenched with 100 μ l/well 1 M H₃PO₄ and the plate read at 490 nm. IC₅₀ values were determined by a four-parameter fit of the competitive displacement ELISA signal. The concentrations of initial stock solutions of bhpBR3 were determined spectrophotometrically as described (Gill and von Hippel, 1989), while those of miniBR3 and BR3 extracellular domain were determined by quantitative amino acid analysis.

BR3-Fc Staining of BAFF-Transfected COS7 Cells

Mutations that disrupt residues within the ²⁸DLLVRH³¹ loop (D26→A26 and L28→A28) were introduced into BR3-Fc cDNA (Yan et al., 2001a) by PCR. COS7 cells transfected with BAFF were stained with 1 μ g/ml BR3-Fc or BR3 A26/A28-Fc. Cells were washed and fixed, and bound receptor detected with Cy3-labeled anti-human IgG Fc (Jackson).

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References

Benhamou, L.E., Cazenave, P.A., and Sarthou, P. (1990). Anti-immunoglobulins induce death by apoptosis in WEHI-231 B lymphoma cells. *Eur. J. Immunol.* 20, 1405–1407.

Bodmer, J.L., Schneider, P., and Tschopp, J. (2002). The molecular architecture of the TNF superfamily. *Trends Biochem. Sci.* 27, 19–26.

Caamano, J.H., Rizzo, C.A., Durham, S.K., Barton, D.S., Raventos-Suarez, C., Snapper, C.M., and Bravo, R. (1998). Nuclear factor (NF)- κ B2 (p100/p52) is required for normal splenic microarchitecture and B cell-mediated immune responses. *J. Exp. Med.* 187, 185–196.

Cheema, G.S., Roschke, V., Hilbert, D.M., and Stohl, W. (2001). Elevated serum B lymphocyte stimulator levels in patients with systemic immune-based rheumatic diseases. *Arthritis Rheum.* 44, 1313–1319.

Cochran, A.G., Tong, R.T., Starovasnik, M.A., Park, E.J., McDowell, R.S., Theaker, J.E., and Skelton, N.J. (2001). A minimal peptide scaffold for β -turn display: optimizing a strand position in disulfide-cyclized β -hairpins. *J. Am. Chem. Soc.* 123, 625–632.

Franzoso, G., Carlson, L., Poljak, L., Shores, E.W., Epstein, S., Leonard, A., Grinberg, A., Tran, T., Scharton-Kersten, T., Anver, M., et al. (1998). Mice deficient in nuclear factor (NF)- κ B/p52 present with defects in humoral responses, germinal center reactions, and splenic microarchitecture. *J. Exp. Med.* 187, 147–159.

Ghosh, S., and Karin, M. (2002). Missing pieces in the NF- κ B puzzle. *Cell* 109, S81–S96.

Gill, S.C., and von Hippel, P.H. (1989). Calculation of protein extinction coefficients from amino acid sequence data. *Anal. Biochem.* 182, 319–326.

Goodnow, C.C. (1992). Transgenic mice and analysis of B-cell tolerance. *Annu. Rev. Immunol.* 10, 489–518.

Groom, J., Kalled, S.L., Cutler, A.H., Olson, C., Woodcock, S.A., Schneider, P., Tschopp, J., Cachero, T.G., Batten, M., Wheway, J., et al. (2002). Association of BAFF/BLyS overexpression and altered B cell differentiation with Sjögren's syndrome. *J. Clin. Invest.* 109, 59–68.

Gross, J.A., Johnston, J., Mudri, S., Enselman, R., Dillon, S.R., Madden, K., Xu, W., Parrish-Novak, J., Foster, D., Lofton-Day, C., et al. (2000). TACI and BCMA are receptors for a TNF homologue implicated in B-cell autoimmune disease. *Nature* 404, 995–999.

Gross, J.A., Dillon, S.R., Mudri, S., Johnston, J., Littau, A., Roque, R., Rixon, M., Schou, O., Foley, K.P., Haugen, H., et al. (2001). TACI-Ig neutralizes molecules critical for B cell development and autoimmune disease, impaired B cell maturation in mice lacking BLyS. *Immunity* 15, 289–302.

Hahne, M., Kataoka, T., Schroter, M., Hofmann, K., Imler, M., Bodmer, J.L., Schneider, P., Bormand, T., Holler, N., French, L.E., et al. (1998). APRIL, a new ligand of the tumor necrosis factor family, stimulates tumor cell growth. *J. Exp. Med.* 188, 1185–1190.

Hasbold, J., and Klaus, G.G. (1990). Anti-immunoglobulin antibodies induce apoptosis in immature B cell lymphomas. *Eur. J. Immunol.* 20, 1685–1690.

Hertz, M., and Nemazee, D. (1998). Receptor editing and commitment in B lymphocytes. *Curr. Opin. Immunol.* 10, 208–213.

Humke, E.W., Shriver, S.K., Starovasnik, M.A., Fairbrother, W.J., and Dixit, V.M. (2000). ICEBERG: a novel inhibitor of interleukin-1 β generation. *Cell* 103, 99–111.

Hymowitz, S.G., Christinger, H.W., Fuh, G., Ultsch, M., O'Connell, M., Kelley, R.F., Ashkenazi, A., and de Vos, A.M. (1999). Triggering cell death: the crystal structure of Apo2L/TRAIL in a complex with death receptor 5. *Mol. Cell* 4, 563–571.

Kaisho, T., Takeda, K., Tsujimura, T., Kawai, T., Nomura, F., Terada, N., and Akira, S. (2001). I κ B kinase α is essential for mature B cell development and function. *J. Exp. Med.* 193, 417–426.

Karpusas, M., Cachero, T.G., Qian, F., Boriack-Sjodin, A., Mullen, C., Strauch, K., Hsu, Y.M., and Kalled, S.L. (2002). Crystal structure of extracellular human BAFF, a TNF family member that stimulates B lymphocytes. *J. Mol. Biol.* 315, 1145–1154.

Khare, S.D., Sarosi, I., Xia, X.Z., McCabe, S., Miner, K., Solovyev, I., Hawkins, N., Kelley, M., Chang, D., Van, G., et al. (2000). Severe B cell hyperplasia and autoimmune disease in TALL-1 transgenic mice. *Proc. Natl. Acad. Sci. USA* 97, 3370–3375.

Klinman, N.R. (1996). The "clonal selection hypothesis" and current concepts of B cell tolerance. *Immunity* 5, 189–195.

- Laskowski, R.A., MacArthur, M.W., Moss, D.S., and Thornton, J.M.P. (1993). A program to check the stereochemical quality of protein structures. *J. Appl. Crystallogr.* 26, 283–291.
- Liu, Y., Xu, L., Opalka, N., Kappler, J., Shu, H.B., and Zhang, G. (2002). Crystal structure of sTALL-1 reveals a virus-like assembly of TNF family ligands. *Cell* 108, 383–394.
- Locksley, R.M., Killeen, N., and Lenardo, M.J. (2001). The TNF and TNF receptor superfamilies: integrating mammalian biology. *Cell* 104, 487–501.
- Mackay, F., Woodcock, S.A., Lawton, P., Ambrose, C., Baetscher, M., Schneider, P., Tschoep, J., and Browning, J.L. (1999). Mice transgenic for BAFF develop lymphocytic disorders along with autoimmune manifestations. *J. Exp. Med.* 190, 1697–1710.
- Moore, P.A., Belvedere, O., Orr, A., Pieri, K., LaFleur, D.W., Feng, P., Soppet, D., Charters, M., Gentz, R., Parmelee, D., et al. (1999). BLyS: member of the tumor necrosis factor family and B lymphocyte stimulator. *Science* 285, 260–263.
- Naismith, J.H., and Sprang, S.R. (1998). Modularity in the TNF-receptor family. *Trends Biochem. Sci.* 23, 74–79.
- Oren, D.A., Li, Y., Volovik, Y., Morris, T.S., Dharia, C., Das, K., Galperina, O., Gentz, R., and Arnold, E. (2002). Structural basis of BLyS receptor recognition. *Nat. Struct. Biol.* 9, 288–292.
- Russell, S.J., and Cochran, A.G. (2000). Designing stable β -hairpins: energetic contributions from cross-strand residues. *J. Am. Chem. Soc.* 122, 12600–12601.
- Schiemann, B., Gommerman, J.L., Vora, K., Cachero, T.G., Shulga-Morskaya, S., Dobles, M., Frew, E., and Scott, M.L. (2001). An essential role for BAFF in the normal development of B cells through a BCMA-independent pathway. *Science* 293, 2111–2114.
- Schneider, P., MacKay, F., Steiner, V., Hofmann, K., Bodmer, J.L., Holler, N., Ambrose, C., Lawton, P., Bixler, S., Acha-Orbea, H., et al. (1999). BAFF, a novel ligand of the tumor necrosis factor family, stimulates B cell growth. *J. Exp. Med.* 189, 1747–1756.
- Seftleben, U., Cao, Y., Xiao, G., Greten, F.R., Krahn, G., Bonizzi, G., Chen, Y., Hu, Y., Fong, A., Sun, S.C., et al. (2001). Activation by IKK α of a second, evolutionary conserved, NF- κ B signaling pathway. *Science* 293, 1495–1499.
- Starovasnik, M.A., Skelton, N.J., O'Connell, M.P., Kelley, R.F., Reilly, D., and Fairbrother, W.J. (1996). Solution structure of the E-domain of Staphylococcal protein A. *Biochemistry* 35, 15558–15569.
- Thompson, J.S., Bixler, S.A., Qian, F., Vora, K., Scott, M.L., Cachero, T.G., Hession, C., Schneider, P., Sizing, I.D., Mullen, C., et al. (2001). BAFF-R, a newly identified TNF receptor that specifically interacts with BAFF. *Science* 293, 2108–2111.
- Xiao, G., Harhaj, E.W., and Sun, S.C. (2001). NF- κ B-inducing kinase regulates the processing of NF- κ B p100. *Mol. Cell* 7, 401–409.
- Xu, S., and Lam, K.P. (2001). B-cell maturation protein, which binds the tumor necrosis factor family members BAFF and APRIL, is dispensable for humoral immune responses. *Mol. Cell. Biol.* 21, 4067–4074.
- Yamada, T., Mitani, T., Yorita, K., Uchida, D., Matsushima, A., Iwamasa, K., Fujita, S., and Matsumoto, M. (2000). Abnormal immune function of hemopoietic cells from alymphoplasia (aly) mice, a natural strain with mutant NF- κ B-inducing kinase. *J. Immunol.* 165, 804–812.
- Yan, M., Marsters, S.A., Grewal, I.S., Wang, H., Ashkenazi, A., and Dixit, V.M. (2000). Identification of a receptor for BLyS demonstrates a crucial role in humoral immunity. *Nat. Immunol.* 1, 37–41.
- Yan, M., Brady, J.R., Chan, B., Lee, W.P., Hsu, B., Harless, S., Cancro, M., Grewal, I.S., and Dixit, V.M. (2001a). Identification of a novel receptor for B lymphocyte stimulator that is mutated in a mouse strain with severe B cell deficiency. *Curr. Biol.* 11, 1547–1552.
- Yan, M., Wang, H., Chan, B., Roose-Girma, M., Erickson, S., Baker, T., Tumas, D., Grewal, I.S., and Dixit, V.M. (2001b). Activation and accumulation of B cells in TACI-deficient mice. *Nat. Immunol.* 2, 638–643.
- Yin, L., Wu, L., Wesche, H., Arthur, C.D., White, J.M., Goeddel, D.V., and Schreiber, R.D. (2001). Defective lymphotoxin- β receptor-induced NF- κ B transcriptional activity in NIK-deficient mice. *Science* 291, 2162–2165.
- Zhang, J., Roschke, V., Baker, K.P., Wang, Z., Alarcon, G.S., Fessler, B.J., Bastian, H., Kimberly, R.P., and Zhou, T. (2001). Cutting edge: a role for B lymphocyte stimulator in systemic lupus erythematosus. *J. Immunol.* 166, 6–10.

Accession Number

The structure of bhpBR3 will be available from the RCSB Protein Data Bank under ID code 1MPV.

8.1 Nonclinical Pharmacology

8.1.1 Summary

The nonclinical pharmacology program for BLyS has been designed to assess the *in vitro* and *in vivo* pharmacological action of BLyS with respect to B cell proliferation, immunoglobulin production, and enhancement of humoral response to foreign antigens. The effects of differing routes of administration, doses, and dosing schedules have also been studied. The findings from the nonclinical pharmacology studies are summarized below.

From *in vitro* pharmacology studies:

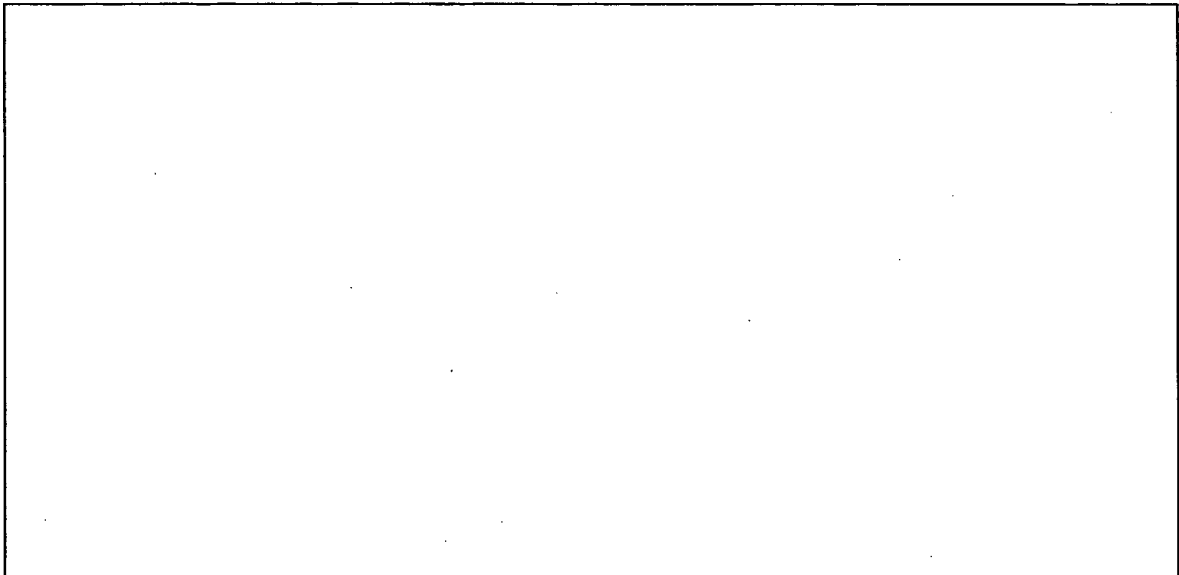
- BLyS induces mouse, monkey, and human B cell proliferation.
- BLyS enhances IgA, IgG and IgM secretion by B lymphocytes.
- BLyS receptor is expressed on mature B cells.

From *in vivo* pharmacology studies:

- BLyS administration has been shown to be well-tolerated and safe.
- BLyS produces increased spleen weight, B lymphocyte hyperplasia, and increases in B cell representation in the spleen and peripheral blood.
- Pharmacologic activity is seen following both subcutaneous and intravenous administration.
- BLyS induces dose- and schedule-dependent increases in serum IgM, IgG, IgA, and IgE and secretory IgA concentrations.
- BLyS enhances antigen-specific Ig responses.
- The *in vitro* findings of BLyS are predictive of the *in vivo* pharmacology and B cell specificity of BLyS.

Taken together, the *in vivo* and *in vitro* pharmacological evaluation of BLyS demonstrates that it stimulates both B cell proliferation and immunoglobulin secretion. The biological profile of BLyS suggests that it may have therapeutic benefit in the treatment of immunodeficiency disorders characterized by low or absent serum Ig. For example, the clinical benefit of BLyS will

be realized if it is able to increase serum Ig levels, thereby reducing the reliance on intravenous administration of pooled human Ig products (IVIG). Additionally, other immunological activities of BLyS (*e.g.*, increasing secretory IgA production and/or enhancement of active humoral immunity) may further reduce the risk of infection.



The primary nonclinical pharmacology studies are summarized in Table 2

Table 2 Summary of Primary Pharmacology of BlyS

Report Number	Experimental Design or Model	Species, Strain, Gender, total number of animals	BlyS Dose and Schedule	Route of Administration or <i>In Vitro</i> Study	Major Findings
	Human tonsillar B lymphocyte proliferation assay	None, <i>in vitro</i> studies	BlyS 0.056 – 10000 ng/mL	<i>in vitro</i>	<ul style="list-style-type: none"> In the presence of SAC, human recombinant BlyS induced a concentration-dependent increase in proliferation of tonsillar B lymphocytes.
	Proliferation of Cynomolgus splenocytes	None, <i>in vitro</i> studies	BlyS 0.46 – 1000 ng/mL	<i>in vitro</i>	<ul style="list-style-type: none"> In the presence of SAC, human recombinant BlyS induced a concentration-dependent increase in proliferation of Cynomolgus splenocytes, thus demonstrating cross species activity.
	Proliferation of BALB/c mouse splenocytes	None, <i>in vitro</i> studies	BlyS 0.04 – 33.3 ng/mL	<i>in vitro</i>	<ul style="list-style-type: none"> In the presence of SAC, human recombinant BlyS induced a concentration-dependent increase in proliferation of BALB/c mouse splenocytes, thus demonstrating cross species activity.
	Effects of BlyS on human tonsillar B lymphocyte Ig secretion	None, <i>in vitro</i> studies	BlyS 100 ng/mL	<i>in vitro</i>	<ul style="list-style-type: none"> Human recombinant BlyS enhanced IgA, IgM, and IgG secretion by human tonsillar B lymphocytes.

Table 2 Summary of Primary Pharmacology of BLYS (continued)

Report Number	Experimental Design or Model	Species, Strain, Gender, total number of animals	BLYS Dose and Schedule	Route of Administration or <i>In Vivo</i> Study	Major Findings
	Effects of BLYS on PBMC from CVID patients	None, <i>ex vivo</i> studies	BLYS HGS (100 ng/mL)	<i>ex vivo</i>	<ul style="list-style-type: none"> • B-lymphocytes from CVID patients express BLYS-R at levels comparable to those observed for normal controls. • Among normal volunteers, <i>ex vivo</i> BLYS responsiveness is manifested as increases in secreted IgA, IgM, and IgG. • Among CVID patients, BLYS responsiveness is primarily manifested as an increase in secreted IgM (7/10 responders), with less frequent increases observed in IgA (3/10 responders) and IgG (3/10 responders). Interestingly, the magnitude of the BLYS-induced IgM secretion is generally greater in CVID than among normal controls. • The frequency of peripheral blood B cells in PBMC from CVID patients varies from 0.05% to 19.17%. <i>Ex vivo</i> BLYS responsiveness among CVID samples does not correlate with peripheral B cell representation or BLYS-R expression. However, it should be noted that analyses of patient samples containing fewer than 1% CD19+ peripheral B cells were more difficult to interpret due to insufficient cell numbers and greater variability in Ig secretion assays. • <i>Ex vivo</i> BLYS responsiveness requires a co-stimulatory signal delivered by PWM or SAC.

Table 2 Summary of Primary Pharmacology of BLyS (continued)

<u>Report Number</u>	<u>Experimental Design or Model</u>	<u>Species, Strain, Gender, total number of animals</u>	<u>BLyS Dose and Schedule</u>	<u>Route of Administration or In Vitro Study</u>	<u>Major Findings</u>
	Effects of ip administration of TL7 (BLyS)	Mouse BALB/c female 27 total (3 buffer controls)	0.08, 0.8, 2, 8 mg/kg Twice daily for 2 or 4 days	intraperitoneal	<ul style="list-style-type: none"> • TL7 (BLyS) administration specifically increases the proportion of mature [CD45R(B220)+], ThB (Ly6D)+ B cells. (Days, DOSE?) • BLyS administration resulted in an increase in serum IgA and IgM concentrations. • Significantly increased serum IgM, IgG1, IgG3, and IgA concentrations are observed following 10 days of BLyS administration over a two week period; all Ig assessed are significantly increased, including IgG2a and IgG2b, following 14 days of BLyS administration. • The Ig increases observed are proportional to the relative serum concentration of each isotype. • The minimum schedule of administration resulting in significant increases in Ig concentrations is 5 consecutive days for 2 consecutive weeks separated by 2 treatment-free days. Less frequent exposures were either ineffective or elicited minimal responses.
	Effects of BLyS dosing schedule on serum Ig concentrations	Mouse BALB/c 50 total (10 buffer controls)	Days 0-13 Days 0-4, 7-11 Days 0, 2, 4, 6, 8, 10, 12 Days 0, 4, 8, 13 Days 0-4, 7-11, 14-16 Days 0-4, 7-11, 14-16 Daily	subcutaneous	<ul style="list-style-type: none"> • BLyS enhances in vivo humoral immune responses of the IgA and IgM isotypes to the polysaccharide antigens contained in the Pneumovax-23 vaccine. These findings suggest that BLyS may function as an adjuvant by enhancing ongoing immune responses.
	Effects of BLyS on Pneumovax-23-specific humoral immune responses	Mouse BALB/c 35 total (5 buffer controls)	Days 0-10 Daily	subcutaneous	<ul style="list-style-type: none"> • These experiments demonstrate that BLyS administration during the course of a DNP-specific immune response enhances the titers of serum IgM and IgG that bind the antigen.
	Effects of BLyS on DNP-specific serum Ig responses	Mouse BALB/c 30 total (5 buffer controls)	Days 0-14 Daily	intraperitoneal	

Pharmacology and Toxicology

Table 2 Summary of Primary Pharmacology of BlyS (continued)

Report Number	Experimental Design or Model	Species, Strain, Gender, total number of animals	BlyS Dose and Schedule	Route of Administration or <i>In Vitro</i> Study	Major Findings
	Effects of ip administration of TL7 (BlyS)	Mouse BALB/c female 27 total (3 buffer controls)	0.08, 0.8, 2, 8 mg/kg Twice daily for 2 or 4 days	intraperitoneal	<ul style="list-style-type: none"> • TL7 (BlyS) administration specifically increases the proportion of mature [CD45R(B220)⁺, ThB (Ly6D)⁺ B cells. (Days, DOSE?) • BlyS administration resulted in an increase in serum IgA and IgM concentrations. • Significantly increased serum IgM, IgG1, IgG3, and IgA concentrations are observed following 10 days of BlyS administration over a two week period; all Ig assessed are significantly increased, including IgG2a and IgG2b, following 14 days of BlyS administration. • The Ig increases observed are proportional to the relative serum concentration of each isotype. • The minimum schedule of administration resulting in significant increases in Ig concentrations is 5 consecutive days for 2 consecutive weeks separated by 2 treatment-free days. Less frequent exposures were either ineffective or elicited minimal responses.
	Effects of BlyS dosing schedule on serum Ig concentrations	Mouse BALB/c 50 total (10 buffer controls)	Days 0-13 Days 0-4, 7-11 Days 0, 2, 4, 6, 8, 10, 12 Days 0, 4, 8, 13 Days 0-4, 7-11, 14-16 Days 0-4, 7-11, 14-16 Daily	subcutaneous	<ul style="list-style-type: none"> • BlyS enhances <i>in vivo</i> humoral immune responses of the IgA and IgM isotypes to the polysaccharide antigens contained in the Pneumovax-23 vaccine. These findings suggest that BlyS may function as an adjuvant by enhancing ongoing immune responses.
	Effects of BlyS on Pneumovax-23-specific humoral immune responses	Mouse BALB/c 35 total (5 buffer controls)	Days 0-10 Daily	subcutaneous	<ul style="list-style-type: none"> • These experiments demonstrate that BlyS administration during the course of a DNP-specific immune response enhances the titers of serum IgM and IgG that bind the antigen.
	Effects of BlyS on DNP- specific serum Ig responses	Mouse BALB/c 30 total (5 buffer controls)	Days 0-14 Daily	intraperitoneal	<ul style="list-style-type: none"> • These experiments demonstrate that BlyS administration during the course of a DNP-specific immune response enhances the titers of serum IgM and IgG that bind the antigen.

Pharmacology and Toxicology

Table 2 Summary of primary pharmacology of BlyS (continued)

Report Number	Experimental Design or Model	Species, Strain, Gender, total number of animals	BlyS Dose and Schedule	Route of Administration or <i>In Vitro</i> Study	Major Findings
	Effects of BlyS on serum immunoglobulins	Mouse, BALB/c Female 96 total (32 buffer control)	0.3, 3.0 mg/kg Day 5 Necropsy Day 1 Days 1-2 Days 1-3 Days 1-4 Daily Day 15 Necropsy Days 1-2 Days 1-4 Days 1-9 Days 1-15	subcutaneous	<ul style="list-style-type: none"> Short term dosing of at least 3 days duration is required to increase serum IgA levels, the only Ig class that was increased at the Day 5 necropsy. Of the eight tested regimens, only long term dosing (<i>i.e.</i>, 14 days) increased serum concentrations of all Ig classes (IgG, IgM, IgA, and IgE). The effects of shorter term dosing (2, 4, or 9 days) on serum Ig concentrations were not apparent at day 15 necropsy (except for IgM after 9 days of dosing). It is likely that the effects of BlyS on serum Ig production are reversible.
	Effects of BlyS on immune system of SCID mice	Mouse, BALB/c SCID Female 20 total	1 mg/kg Days 1-14 Daily	subcutaneous	<ul style="list-style-type: none"> In SCID mice, BlyS treatment had no significant effects on serum immunoglobulin concentrations or on spleen weight. B and T lymphocytes could not be quantitated by FACS. In BALB/c mice, BlyS treatment caused predicted increases in spleen weight, serum immunoglobulin concentrations, and splenic B cell representation and the new finding of increases in B cell representation in the blood. Subcutaneous and intravenous dosing produce similar increases in Ig concentrations. BlyS increases serum IgG, IgM, IgE, IgA and salivary IgA concentrations in a dose-dependent manner. 14-day dosing produces greater increases in Ig concentrations than 4-day dosing.
	Comparison of BlyS effects on serum immunoglobulin levels	Mouse BALB/c Female 72 total (24 buffer controls)	0.3, 3 mg/kg Days 1-4 Days 1-14 Daily	subcutaneous intravenous	<ul style="list-style-type: none"> Subcutaneous and intravenous dosing produce similar increases in Ig concentrations. BlyS increases serum IgG, IgM, IgE, IgA and salivary IgA concentrations in a dose-dependent manner. 14-day dosing produces greater increases in Ig concentrations than 4-day dosing.

Pharmacology and Toxicology

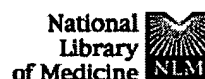
Table 2 Summary of primary pharmacology of BLyS (continued)

Report Number	Experimental Design or Model	Species, Strain, Gender, total number of animals	BLyS Dose and Schedule	Route of Administration or <i>In Vitro</i> Study	Major Findings
	Effect of subcutaneously administered BLyS on serum IgA, IgM, and IgG, as well as splenic cell populations in mice	Mouse BALB/c Female 80 total (32 buffer controls)	0.3, 3.0 mg/kg Day 5 Necropsy Day 1 Days 1-2 Days 1-3 Days 1-4 Daily Day 16 Necropsy Days 1-2 Days 1-4 Days 1-9 Days 1-15 Daily	subcutaneous	<ul style="list-style-type: none"> • BLyS increased serum IgA, IgG, and IgM concentrations in a dose (IgA) and dosing frequency-dependent manner. • Daily dosing for 15 days produced a greater effect on serum Ig concentrations compared to the 4-day regimen. • IgA was the only Ig significantly increased on Day 5, IgM and IgG, but not IgA, were increased on Day 16. • BLyS increased splenic weight, but had not effect on thymic weight. • FACS analyses suggested BLyS expanded splenic B cell populations (along with the number of more mature B cells). FACS analyses also suggested the relative number of CD8+ T cells were reduced. These alterations in splenic B and T cell populations were rapidly induced after short term dosing with BLyS. • The above listed effects of BLyS were reversible with drug withdrawal. • Four days of dosing with BLyS at 3 mg/kg is insufficient to increase salivary IgA in mice • Under these same conditions, BLyS did significantly increase spleen weights, although neither IgA nor IgM concentrations were significantly elevated in the serum.
	Effect of subcutaneously administered BLyS on salivary IgA levels	Mouse BALB/c Female 12 total (6 buffer controls)	3.0 mg/kg Day 1-4 Daily	subcutaneous	<ul style="list-style-type: none"> • BLyS may confer partial protection against a lethal <i>Staphylococcus aureus</i> infection.
	Effects of BLyS on mouse survival after a lethal <i>Staphylococcus aureus</i> challenge	Mouse NIH/SWISS Female 50 total (10 buffer control)	20 µg/mouse Days 1-7 Daily Cyclophosphamide 250 mg/kg, Day 0 <i>S. aureus</i> 410 cfu, Day 4	intraperitoneal	

Pharmacology and Toxicology

Table 2 Summary of Primary Pharmacology of BlyS continued

Report Number	Experimental Design or Model	Species, Strain, Gender, total number of animals	BlyS Dose and Schedule	Route of Administration or <i>In Vitro</i> Study	Major Findings
	Effects of BlyS on mouse survival after a lethal <i>Staphylococcus aureus</i> challenge	Mouse NIH/SWISS Female 50 total (10 buffer control)	20 µg/mouse Days -3 to Day 3 Daily Cyclophosphamide 250 mg/kg, Day 0 <i>S. aureus</i> 410 cfu, Day 4	intraperitoneal	<ul style="list-style-type: none">BlyS treatment in this study did not improve the survival of immunocompromised mice when they were challenged with a lethal dose of <i>Staphylococcus aureus</i>.



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We have previously shown that retinoids can induce differentiation of B cells in vitro as well as in vivo in patients with common variable immunodeficiency (CVI). While changes were observed over 1 week when retinoic acid (RA) was added to CVI hybridoma cells in vitro, maturation of the patients' B-cell compartment in vivo occurred only after 4 months of drug administration. We have now performed a 64-week open trial of oral 13-cis RA in five patients to see if prolonged treatment would result in continued improvement in their humoral immune compartment. In this trial, drug was given for 32 weeks followed by a 32-week wash-out period. During the treatment, the patients showed changes in a variety of parameters indicating an alteration towards normal of their humoral immune systems. This change included a fall in the elevated circulating interleukin-6 (IL-6) levels, a more normal display of B-cell surface markers (L-selectin and CD20), a decrease in B-cell size, and improved in vitro and in vivo B-cell function. In order to determine if VH gene use was affected by the retinoid treatment, VH gene expression in the CVI patients was characterized. Results showed an unusual predominance of non-mutated VH gene sequences, representative of cells that are recent bone marrow emigrants. While no common pattern of change occurred in VH gene segment use in the patients while on retinoid therapy, large-scale (> 10-fold) changes in the expression of these genes were observed in each individual over time. Taken together, these results provide multiple lines of evidence that 13-cis RA promotes maturation of B cells in patients with CVI. However, the effect appears to be partial, such that stimuli in addition to 13-cis RA will be necessary to provide for further B-cell differentiation in order to achieve normalization of humoral immunity.

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Common variable immunodeficiency (CVI) is a primary immunodeficiency characterized by deficient antibody production. The cause of this immunodeficiency is unknown; several in vitro studies have revealed a significant number of alterations that could explain the hypogammaglobulinemia present in this syndrome. Among those described are primary B cell alterations, numerical and functional T cell abnormalities, and defects in the interaction between accessory cells. The alteration typical of CVI is the failure of B lymphocytes to differentiate from antibody-producing cells, resulting in deficient immunoglobulin secretion. Among the T cell abnormalities described are a diminished proliferative response to mitogens and antigens, alterations in the level of production of several cytokines, especially reduction in the production of IL-2, diminished antigen-specific T cells and increase basal apoptosis after stimulation. Antigen presenting cells, monocytes and dendritic cells can also present alterations and contribute to deficient antigen response. The clinical manifestations of these patients is variable; most present recurrent bacterial infections due to encapsulated bacteria, especially sinusitis, otitis, bronchitis, and pneumonias. A few patients can present mycobacterial or fungal infection and occasionally *Pneumocystis carinii*. Viral infection is uncommon in these patients although some suffer recurrent herpes zoster infection. Clinical features of septicemia and central nervous system infections are less frequent. The incidence of digestive tract infections in these patients is high. The most common cause of diarrhea is *Giardia lamblia*; *Salmonella*, *Shigella* and *Campylobacter* are also common pathogens. Autoimmune disease is also more prevalent in these patients than in the general population. The most frequently associated diseases are hemolytic anemia, idiopathic thrombocytopenic purpura and autoimmune neutropenia. Cancer is also frequently associated with CVI, the most common forms being lymphoproliferative syndromes, especially non-Hodgkin's lymphoma. Granulomas are a unusual manifestation in some patients with CVI; their localization varies

but the most commonly affected organs are the spleen and lungs. Some authors have compared these granulomas with those characterizing sarcoidosis, especially when appearing in the lung. Diagnosis of CVI is usually by exclusion of other diseases, such as cystic fibrosis, immotile cilia syndrome or allergic processes. CVI should be suspected in all patients with recurrent bacterial infections especially those localized in the respiratory tract. Other primary immunodeficiencies which present clinical findings similar to CVI and which should be ruled out are selective IgG subclass deficiency, IgA deficiency and selective deficiency in the response to polysaccharide antigens with normal immunoglobulin levels. The serum hypogammaglobulinemia present in all patients with CVI provides the diagnostic key. The age at which clinical manifestations appear, the absence of familial antecedents and the presence of circulating B lymphocytes form the basis of the differential diagnosis between X-linked agammaglobulinemia and autosomal recessive forms. The treatment of choice of patients with CVI is treatment with human gamma-globulin. Currently, the most common route of administration is intravenous; these molecules have a half-life of approximately 21 days and a high degree of safety concerning the possible transmission of viral infections. Adverse reactions are generally few and clinically unimportant. The most frequently used doses oscillate between 200 and 400 mg/kg body weight every 2-4 weeks. Both the dose and its frequency should be personalized for each patient. Early diagnosis of patients with CVI, application of treatment with appropriate antibiotics for infections and treatment with gamma-globulins prevent long-term complications of this disease and dramatically improve the quality of life and life expectancy of these patients.

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(TNF), and interferons. These cause fever and further augment the inflammatory reaction by attracting additional cells, augmenting the activity of these cells, and inducing vasodilatation (see Chapter 7).

Fever

Elevation of body temperature (fever) in response to infection is nearly universal in humans and other animals; this response has been highly conserved during evolution. It is therefore reasonable to conclude that fever is an important host defense mechanism. However, this contention has been difficult to prove, except for poikilothermic animals such as lizards. The main problem in proving the role of fever in antimicrobial defense in homoiothermic animals has been in dissociating the effects of the endogenous pyrogens (IL-1, TNF) that produce fever from the complex effects of fever per se. Fever has a salutatory effect on the course of infection, whereas hypothermia has deleterious effects. Although these data would argue for not reducing fever in patients with infections (eg, through tepid sponging or antipyretic drugs such as aspirin), high fever itself may be deleterious. In addition, the role of fever as a host defense mechanism is probably adjunctive rather than central, and it becomes insignificant if effective antimicrobial chemotherapy is being administered.

IMMUNOLOGIC DEFENSES AGAINST INFECTION

Immunologic defenses are, by definition, those host defense mechanisms which have specificity toward the invading pathogen and which are augmented on second and subsequent exposures. The specificity of the host response to invading microorganisms is determined primarily by immunoglobulins and T lymphocytes. However, the response frequently requires recruitment of otherwise nonspe-

cific components such as complement and phagocytic cells.

Antibody-Mediated Host Defenses

Antibodies serve a variety of important host defense functions, both alone and in conjunction with nonspecific effectors (Table 49-4) (see Chapter 9). Functions of antibodies include neutralization of the biologic activity of bacterial toxins (the mechanism by which tetanus and diphtheria toxoid vaccines protect against disease), inhibition of enzyme activity (eg, the neuraminidase of influenza virus), blocking of the adherence of bacteria to mucosal surfaces, and inhibition of the growth of some prokaryotes such as *Mycoplasma*. Viruses may be neutralized in the presence of antibody alone, but many enveloped viruses are neutralized more efficiently if complement is also present. Although in vitro lysis of virus-infected cells by specific antibody and complement has been documented, the overall role of this phenomenon in host defense is unclear. Opsonization—preparing material for ingestion by phagocytic cells—also may occur with antibody alone, although the combination of antibody and complement usually increases the efficiency of ingestion. Killing of gram-negative bacteria by IgM antibody has an absolute requirement for complement.

T Lymphocyte-Mediated Host Defenses

Although T lymphocytes play a central and crucial role in the generation of the immune response to invading microorganisms (see also Chapter 5), their role is predominantly one of recruiting, facilitating, and augmenting other effectors, rather than of directly attacking the pathogens themselves. Viruses are the major exception to this generalization, as the T lymphocyte-mediated attack on virus-infected cells constitutes a major host defense against established viral infection. The specific T cell response to virus-

infected cells is mediated by CD8 cytotoxic T lymphocytes (CTLs); in addition, for some viral infections (eg, by herpes simplex virus) NK cells and macrophages are important in recovery from infection. Secretion of lymphokines, especially gamma interferon, by immune lymphocytes is responsible for augmented NK and CTL activity. Lymphokines also activate macrophages, which constitute a major host defense against many bacterial, fungal, and parasitic infections. Interferons produced by virus-infected cells that are not a part of the immune system (eg, alpha interferon from fibroblasts) have a direct antiviral effect, but they also augment NK cell and macrophage function. A possible direct role for T cells in antibacterial defense has been suggested by recent studies showing specific lysis of *Listeria monocytogenes*-infected macrophages by sensitized T lymphocytes.

Complement

Complement acts by inactivating microorganisms and by facilitating phagocytosis (opsonization); in both roles it is often assisted by antibody (Table 49-4) (see Chapter 14). In addition, complement breakdown products induce vasodilation and are chemotactants. The alternative complement pathway alone can be stimulated to kill some gram-negative bacteria and inactivate some viruses in the absence of antibodies. Specific antibody, however, is required for activation of the classic complement pathway, which plays an important role in host defenses to bacterial and other infections (Table 49-4). Early in infection, prior to synthesis of specific antibodies, the ability of the alternative complement pathway to nonspecifically opsonize or kill certain bacteria may be critical to recovery from infections.

Phagocytic Cells

Phagocytic cells fulfill a number of important functions in host defense (Table 49-5) (see Chapter 12). They subserve nonspecific roles such as phagocytosis and secretion of monokines and enzymes, but these functions are often augmented by lymphokines

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IMMUNOPATHOLOGY

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Table 49-4. Principal antibody-mediated host defense.

Immunologic Function	Pathogens Affected ¹	Principal Antibody Classes Involved	Nonspecific Cotactors Required
Opsonization	V, B, F	IgG, IgM	Phagocytic cells and complement (in some cases)
Neutralization	V, B ²	IgG, IgM, IgA	Complement (in some cases)
Inhibition of binding	B, F(?)	IgA	None
Bacteriolysis	B	IgM	Complement
Toxin neutralization	B	IgG	None

Table 49-5. Functions of phagocytic cells thought to be important in host defenses.

Chemotaxis
Phagocytosis
Nonfacilitated
-Facilitated (opsonization)
Killing
Intracellular

these agents on a compromised mucosal immune system could result in an increased incidence of infection, autoantibodies, autoimmune disease, and cancer. Recently, an increased prevalence of HLA-A1, -B8, and -Dw3 has been found in patients with IgA deficiency and autoimmune disease.

Lymphocyte culture studies in IgA-deficient patients have demonstrated that IgA cells synthesize but fail to secrete IgA. Some individuals have suppressor T cells that selectively inhibit IgA production by normal lymphocytes.

Acquired IgA deficiency and susceptibility to sinopulmonary tract infections occur frequently in patients treated with phenytoin or penicillamine. In at least some instances, the IgA level returns to normal when the drug therapy is stopped.

Clinical Features

A. Symptoms and Signs:

1. Recurrent sinopulmonary infection—The most frequent presenting symptoms are recurrent sinopulmonary viral or bacterial infections. Patients may occasionally present with recurrent or chronic right middle lobe pneumonia. Pulmonary hemosiderosis occurs with increased frequency and may be erroneously diagnosed as chronic lung infection.

2. Allergy—In surveys of selected atopic populations the prevalence of selective IgA deficiency is 1:400–1:200, compared with a prevalence of 1:800–1:600 in the normal population. Although the reasons for this association are not known, the absence of serum IgA may result in a significant reduction in the amount of antibody competing for antigens capable of combining with IgE. Alternatively, patients who lack IgA in their secretions may more readily absorb allergenic proteins, thereby enhancing the formation of IgE antibodies. Allergic diseases in patients with selective IgA deficiency are often more difficult to control than the same allergies in other patients. Allergic symptoms in these patients may be “triggered” by infection as well as by other environmental agents.

An increase in circulating antibody to bovine proteins, sometimes associated with circulating immune complexes, including complexes with human antibody to bovine immunoglobulin, has been found in patients with selective IgA deficiency. This has been interpreted as providing additional evidence for abnormal gastrointestinal tract absorption. However, removal of cow's milk from the diet is usually not effective in ameliorating symptoms.

A unique form of allergy exists in these patients.

tions. Most patients who have anti-IgA antibodies have not had a history of gamma globulin or blood administration, suggesting that these antibodies are “autoantibodies” or that they arise from sensitization to breast milk, passive transfer of maternal IgA, or cross-reaction with bovine immunoglobulin from ingestion of cow's milk.

3. Gastrointestinal tract disease—An increased prevalence of celiac disease has been noted in patients with selective IgA deficiency. The disease may present at any time and is similar to celiac disease unassociated with IgA deficiency. Intestinal biopsies show an increase in the number of IgM-producing cells. An anti-basement membrane antibody has also been found with increased incidence. Ulcerative colitis and regional enteritis have also been reported in association with selective IgA deficiency. Pernicious anemia has been found in a significant number of patients who also have antibodies to both intrinsic factor and gastric parietal cells.

4. Autoimmune disease—A number of autoimmune disorders are associated with selective IgA deficiency. They include SLE, rheumatoid arthritis, dermatomyositis, pernicious anemia, thyroiditis, Coombs-positive hemolytic anemia, Sjögren's syndrome, and chronic active hepatitis. Although the association of IgA deficiency and certain autoimmune disorders may be fortuitous, the increased prevalence of IgA deficiency in patients with SLE and rheumatoid arthritis (1:200–1:100) is statistically significant.

The clinical presentation of patients with autoimmune disease associated with selective IgA deficiency does not appear to differ significantly from that of individuals with the identical disorder and normal or elevated levels of IgA. Because patients with selective IgA deficiency are capable of making normal amounts of antibody in the other immunoglobulin classes, they usually have the autoantibodies that characterize the specific autoimmune diseases (antinuclear antibody, anti-DNA antibody, antiparietal cell antibody, etc.).

5. Selective IgA deficiency in apparently healthy adults—Patients with selective IgA deficiency are capable of making normal amounts of antibody of the IgG and IgM classes. Many are entirely asymptomatic, although long-term follow-up of some of these patients indicates that they may develop significant disease with time. There are several reasons why some patients remain asymptomatic. A small percentage of patients with selective IgA defi-

ciency may have different exposures to pathogens and noxious agents in the environment.

6. Selective IgA deficiency and genetic factors—Both an autosomal recessive and an autosomal dominant mode of inheritance of IgA deficiency have been postulated. IgA deficiency appears with greater than normal frequency in families with other immunodeficiency disorders such as hypogammaglobulinemia. Partial deletion of the long or short arm of chromosome 18 (18q syndrome) or ring chromosome 18 has been described in selective IgA deficiency. However, many patients with abnormalities of chromosome 18 have normal levels of IgA in their serum. Selective IgA deficiency has been reported in one identical twin but not the other. In a study of familial IgA deficiency, an association with HLA-A2, -B8, and -Dw3 was described. Other studies have shown an increase in association with HLA-A1 and -B8.

7. Selective IgA deficiency and cancer—Selective IgA deficiency has been reported in association with thymoma, reticulum cell sarcoma, and squamous cell carcinoma of the esophagus and lungs. Several patients with IgA deficiency and cancer also had concomitant autoimmune disease and recurrent infection.

8. Selective IgA deficiency and drugs—Phenytoin and other anticonvulsants have been implicated as a possible cause of some cases of selective IgA deficiency or hypogammaglobulinemia, and these patients are frequently symptomatic with recurrent sinopulmonary infections. Withdrawal of the drug does not always result in a return to normal IgA levels. In vitro production of IgA by peripheral blood lymphocytes in these patients may be normal or deficient. Deficient T-cell/B-cell interaction is found in some patients.

B. Laboratory Findings: Selective IgA deficiency is defined as a serum level of IgA below 5 mg/dL, with normal or increased levels of IgG, IgM, IgD, and IgE. Some patients with IgA deficiency may also have IgG2 subclass deficiency. Because there are a number of methods for measuring immunoglobulin levels, each laboratory should establish standards for detection of low IgA levels. B cells from these patients are capable of forming normal amounts of antibody following immunization. In most instances, absence of IgA in the serum is associated with absence of IgA in the secretions and with the presence of normal secretory component. Increased amounts of 7S IgM may be found in the serum and secretions. As discussed above, some pa-

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Cell-mediated immunity may be intact or may be depressed, with negative hypersensitivity skin tests; depressed responses of peripheral blood lymphocytes to PHA and allogeneic cells, and decreased numbers of circulating peripheral blood T cells. The number of B cells in the peripheral blood may be normal or diminished. There is occasionally an increased number of null cells, ie, lymphocytes lacking surface markers for either T or B cells.

Differential Diagnosis

The clinical presentation of patients with X-linked infantile hypogammaglobulinemia and those with acquired hypogammaglobulinemia may be similar. This does not present a major clinical problem. Severe malabsorption in protein-losing enteropathy may cause hypogammaglobulinemia, but these patients always have a concomitant deficiency of serum albumin. Differentiating between protein-losing enteropathy and acquired hypogammaglobulinemia may be difficult under circumstances where protein loss of lymphoid cells. In both groups of patients, antibody responses and cell-mediated immunity may be impaired. When the presenting feature of acquired hypogammaglobulinemia is an autoimmune disease, there may be a delay in recognizing and treating the immune deficiency. In most instances, however, patients with autoimmune disease have normal or elevated immunoglobulin levels. Patients with chronic lung disease should also be investigated for cystic fibrosis, chronic allergy, or antitrypsin deficiency, or immotile cilia syndrome. Patients with HIV infection may occasionally develop hypogammaglobulinemia.

Treatment

The treatment of acquired hypogammaglobulinemia is identical to that of X-linked infantile hypogammaglobulinemia (Table 23-5). Gamma globulin and continuous administration of antibiotics are usually required. Intravenous gamma globulin at doses of 100-200 mg/kg is given once each month. During acute illnesses, gamma globulin can be given weekly or daily. Patients should be monitored at regular intervals with chest x-rays and pulmonary function tests to determine the adequacy of therapy. Pulmonary physical therapy is an essential part of treatment in patients with chronic lung disease.

Respond to dietary restrictions. If the malabsorption is associated with *G lamblia* infection, metronidazole therapy should be used.

Caution should be exercised in the treatment of associated autoimmune disorders. The use of corticosteroids and immunosuppressive agents in a patient with immunodeficiency may result in markedly increased susceptibility to infection. Splenectomy has been used in the treatment of hypogammaglobulinemia and hemolytic anemia, but the mortality rate from overwhelming infection is high.

Complications & Prognosis

Patients with acquired hypogammaglobulinemia may survive to the seventh or eighth decade. Women with this disorder have had normal pregnancies and delivered normal infants (albeit hypogammaglobulinemic until 6 months of age). The major complication is chronic lung disease, which may develop despite adequate gamma globulin replacement therapy. An increased prevalence of malignant disease, including leukemia, lymphoma, and gastric carcinoma, has been observed. Patients who develop acquired T cell deficiencies have increasing difficulty with infection characteristic of both T and B cell deficiencies.

IMMUNODEFICIENCY WITH HYPER-IGM

This syndrome, characterized by an increased level of IgM (ranging from 150 to 1000 mg/dL) associated with a deficiency of IgG and IgA, is relatively rare and in most instances appears to be inherited in a X-linked manner. However, several cases have been reported of an acquired form that affects both sexes. The cause is not known. It has been postulated that in the normal individual there is a sequential development of immunoglobulins, initiated by IgM production and subsequently resulting in the production of IgG and IgA. Arrest in the development of immunoglobulin-producing cells after the formation of IgM-producing cells would be a possible cause. The disorder may be congenital, acquired, or a complication of EBV infection. Inheritance may be X-linked or autosomal dominant or recessive.

Patients present with recurrent pyogenic infections, including otitis media, pneumonia, and septicemia. Some have recurrent neutropenia, hemolytic anemia, or aplastic anemia.

Laboratory evaluation reveals a marked increase in the serum IgM level, with absence of IgG and IgA. Isohemagglutinin titers may be elevated, and

producing plasma cells.

Treatment is similar to that of X-linked infantile hypogammaglobulinemia (Table 23-5). Because so few cases have been reported, it is difficult to determine the prognosis.

SELECTIVE IgA DEFICIENCY

Major Immunologic Features

- IgA level is below 5 mg/dL, with other immunoglobulin levels normal or increased.
- Cell-mediated immunity is usually normal.
- There is increased association with allergies, recurrent sinopulmonary infection, gastrointestinal tract disease, and autoimmune disease.

General Considerations

Selective IgA deficiency is the most common immunodeficiency disorder. The prevalence in the normal population has been estimated to vary between 1:800 and 1:600. Considerable debate exists about whether individuals with selective IgA deficiency are "normal" or have significant associated diseases. Studies of individual patients and extensive studies of large numbers of patients suggest that absence of IgA predisposes to a variety of diseases. The diagnosis of selective IgA deficiency is established by finding a serum IgA level of less than 5 mg/dL.

Immunologic Pathogenesis

The cause of selective IgA deficiency is not known. An arrest in the development of B cells has been suggested on the basis of the observation that these patients have increased numbers of B cells with both surface IgA and IgM or surface IgA and IgD. An associated IgG2 subclass deficiency has been found in some patients, and this has been used to explain the varied clinical manifestations related to antibody deficiency. The presence of normal numbers of circulating IgA-bearing B cells suggests that this disorder is associated with decreased synthesis or release of IgA or impaired differentiation to IgA plasma cells rather than with the absence of IgA B lymphocytes. Utilizing the concept of sequential immunoglobulin production (IgM to IgG to IgA), selective IgA deficiency could result from an arrest in the development of immunoglobulin-producing cells following the normal sequential development of IgM to IgG. The variety of diseases associated with selective IgA deficiency may be the result of enhanced or prolonged exposure to a spectrum of microbial agents and nonreplicating antigens as a consequence of deficient secretory IgA. The continuous assault by